PHARMACOLOGICAL EVALUATION OF GOLD NANOPARTICLES

A Dissertation Submitted to

THE TAMIL NADU Dr. M. G. R. MEDICAL UNIVERSITY

CHENNAI-600 032

In partial fulfillment of the requirement for the award of the Degree of

MASTER OF PHARMACY

IN

PHARMACOLOGY

OCTOBER-2017

DEPARTMENT OF PHARMACOLOGY

KMCH COLLEGE OF PHARMACY,

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Submitted by

Anusree. E

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Under the Guidance of

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This is to certify that the dissertation work entitled **"PHARMACOLOGICAL EVALUATION OF GOLD NANOPARTICLES"** was carried out by **Ms. Anusree. E (Reg. No. 261525803)**. The work mentioned in the dissertation was carried out at the Department of Pharmacology, KMCH College of Pharmacy, Coimbatore, Tamil Nadu, under the guidance of Dr. K. T. Mani Senthil Kumar, M Pharm, Ph. D for the partial fulfillment for the degree of Master of Pharmacy during the academic year 2016-2017 and is forwarded to the Tamil nadu Dr. M. G. R. Medical University, Chennai.

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This research work either in part or full does not constitute any of any thesis / dissertation.

Date:

Place: Coimbatore

Dr. K. T. Mani Senthil Kumar, M Pharm, Ph. D,

DECLARATION

I do here by declare that to the best of my knowledge and belief ,the dissertation work entitled **"PHARMACOLOGICAL EVALUATION OF GOLD NANOPARTICLES''** submitted to the Tamil Nadu Dr. M. G. R. Medical university, Chennai, in the partial fulfillment for the Degree of **Master of Pharmacy** in **Pharmacology,** was carried out at Department of Pharmacology, KMCH College of Pharmacy, Coimbatore under the guidance of Dr. K. T. Mani Senthil Kumar, M. Pharm, Ph. D, during the academic year 2016-2017.

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EVALUATION CERTIFICATE

This is to certify that the work embodied in the thesis entitled **"PHARMACOLOGICAL EVALUATION OF GOLD NANO PARTICLES"** submitted by **Ms**. **Anusree. E (Reg. No:261525803)** to the Tamil Nadu Dr. M.G.R. Medical university, Chennai, in the partial fulfillment for the Degree of **Master of Pharmacy** in **Pharmacology,** is a bonafide research work carried out by the candidate during the academic year 2016-2017 at KMCH College of Pharmacy, Coimbatore, Tamil Nadu and the same was evaluated by us.

Examination Center: KMCH College of Pharmacy, Coimbatore

Date:

Internal Examiner **External Examiner**

Convener of Examination

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Internal Examiner **External Examiner**

Convener of Examination

Certificates

Acknowledgment

Introduction

Review of Literature

Aim and Objectives

Plan of Work

Formulation Profile

Materials and Methods

Results

Discussion

Conclusion

Bibliography

DEDICATED TO ALMIGHTY,

MY BELOVED PARENTS,

BROTHERS, SISTERS

AND MY DEAR FRIENDS

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"Through gratitude comes deep from the heart, if left unexpressed loses its memory, charm and above all, the biggest asset, its beauty"

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LIST OF ABBREVIATIONS

SL NO	ABBREVIATIONS	FULL FORM
1	ALP	Alkaline Phosphate
2	ANOVA	Analysis of Variance
3	DEN	Diethyl Nitrosamine
4	DMSO	Dimethyl sulfoxide
5	DNA	Deoxyribonucleic acid
6	EDTA	Ethylenediaminetetraacetic acid
7	EGFR	Epidermal growth factor receptors
8	ER	Endoplasmic reticulum
9	FBS	Fetal bovine serum
10	GCS	Gold colloid solution.
11	gm	Gram
12	GNP	Gold Nanoparticles
13	HBV	Hepatitis B Virus
14	HCC	Hepatocellular Carcinoma
15	HCL	Hydrochloric Acid
16	HCV	Hepatitis C Virus
17	i.p	Intra peritoneal
18	IL	Interleukin
19	kg	Kilo gram
20	LDH	Lactate dehydrogenase
21	mg/dl	Milligram deci Litre
22	ml	Milli Litre
23	MTT	Microculture tetrazolium Assay
24	NADH	Nicotinamide adenine nucleotide
25	NADPH	Nicotinamide adenine dinucleotide phosphate
26	NCCS	National Centre for Cell Science
27	nm	Nanometer

28	NPs	Nanoparticles
29	p.o	Per Oral
30	PG	Prostaglandin
31	PI	Percentage inhibition
32	ROS	Reactive Oxygen Species
33	SD	Standard Deviation
34	SEM	Standard Error Mean
35	SGOT	Serum Glutamate Oxaloacetate Transaminase
36	SGPT	Serum Glutamate Pyruvate Transaminase
37	TCA	Trichloro Acetic Acid
38	TNF-α	Tumor necrosis factor alpha
39	µg	Micro gram
40	µL	Micro Litre

LIST OF TABLES

LIST OF FIGURES

CONTENTS

1. INTRODUCTION

CANCER

Among the entire diseases, cancer ranks high as a major killer worldwide. Cancers are a large family of diseases that includes the abnormal cell growth with the potential to invade or spread to other parts of the body**.** The term cancer derives from the Greek word (Karkinoma) for crab, by Hippocrates used to describe the appendage-like projections extending from tumours. A tumor is any abnormal proliferation of cells, which may be either benign or malignant. Both benign and malignant tumor attacks surrounding normal tissue and spread throughout the body via the circulatory or lymphatic systems. Cancer refers to a disease of cells that show unlimited proliferation, dedifferentiation, invasiveness and the ability to metastasis .The branch of science dealing with the study of tumours or neoplasms is known as oncology. [1, 2]

The features are common to all types of cancer:

- Abnormal cell growth
- Capacity to invade other tissues
- Capacity to spread to distant organs via blood vessels or lymphatic channels (metastasis)

Cancers can be grouped into 5 main categories based on the type of cell they start in.

- **Carcinoma** – cancer starts in the skin or in tissues that cover internal organs. There are a number of subtypes, including adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, and transitional cell carcinoma
- **Sarcoma** – cancer starts in the connective or supportive tissues such as bone, cartilage, fat, muscle, or blood vessels
- **Leukaemia** – cancer that starts in blood forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and go into the blood
- **Lymphoma and myeloma** – cancers that begin in the cells of the immune system
- **Brain and spinal cord cancers** – these are known as central nervous system cancers. [3]

PATHOPHYSIOLOGY [4]

Cancers, occurs by a sequence of mutations, which causes the variation of the behavior of the cells. Normal cell mutated into cancer cells, reason for the failure of regulation of the cell growth and the carcinogenisis

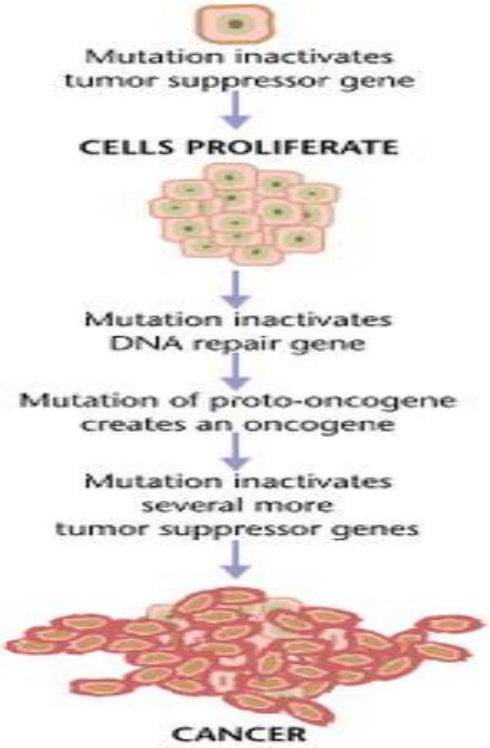


Figure: 1 Pathophysiology of Cancer

SIGNS AND SYMPTOMS

A symptom is a signal that is felt or noticed by the person who has. It may not be easily seen. A sign is a signal that can be seen by the person.

- Fatigue, fever
- Weight changes(loss or gain)
- Yellowing, darkening or redness of the skin, sores that won't heal, or changes to existing moles
- Changes in bowel or bladder habits

- Bowels to anemia or rectal bleeding
- Ulceration can cause bleeding
- A persistent cough or trouble breathing
- Esophageal cancer can cause narrowing of the esophagus difficulty swallowing
- Hoarseness
- Persistent indigestion or discomfort after eating
- Persistent, unexplained muscle or joint pain
- Unexplained bleeding or bruising [5]

LIVER CANCER

Liver cancer, also known as hepatic cancer or primary hepatic cancer. Cancer which has spread from another place to the liver is known as liver metastasis, is more common than that which starts in the liver. The major site of metabolism of ingested materials in liver, it is more susceptible to carcinogenic insult. Moreover, due to the high acceptance of liver, hepatocellular carcinoma is seldom detected at the early stage and once detected treatment has a poor prognosis in most cases. The liver can be affected by primary liver cancer that originates in the liver or by cancer which forms in other parts of the body and then spreads to the liver. The most common cause of liver cancer is cirrhosis which occurs due to hepatitis B, hepatitis C, or alcohol. The viruses induce malignant changes in the cells by affecting gene expression, altering gene methylation and repressing or promoting various cellular signal transduction pathways. Secondary liver cancer occurs as a result of metastasis of cancer from different parts of the body (intestine or pancreas) that drain into the liver via the portal vein or from other cancers. Liver cancer can also form from various other structures within the liver such as the blood vessels, bile duct and the immune cells.

Liver cancers include:

- Hepatocellular carcinoma (HCC)
- Cholangiocarcinoma

Cholangiocarcinoma refers to the cancer of the bile duct. The most frequent liver cancer which comprises 75% of all primary liver cancers is hepatocellular carcinoma.

Hepatocellular carcinoma is a cancer formed by the liver cells, called as hepatocytes that become malignant.

The Hepatocellular carcinoma (HCC) is most common primary malignancy in liver and the third leading cause of cancer deaths worldwide, with few effective therapeutic options for this severe disease. HCC is associated with abdominal pain, abdominal mass, emesis, anaemia, jaundice and fever Most of the HCC appears in cirrhotic livers after years of chronic liver inflammation caused by hepatitis viral infection, alcoholic and non-alcoholic steatohepatitis. [6]

LIVER CANCER RISK FACTORS:

- Hepatitis B virus (HBV)
- Hepatitis C virus (HCV)
- Cirrhosis
- Aflatoxin exposure
- Heavy alcohol drinking
- Tobacco
- Smoking, Monogenic syndromes such as hereditary hemochromatosis and α-1 antitrypsin deficiency. [7]

SYMPTOMS

- Pain in the upper right part of your belly
- A lump or feeling of heaviness in your upper belly
- Bloating or swelling in your belly
- Loss of appetite and feelings of fullness
- Weight loss
- Weakness or deep fatigue
- Nausea and vomiting
- Yellow skin and eyes
- Pale, chalky bowel movements and dark urine
- Fevers [8]

DIAGNOSIS

- Abdominal CT scan
- Abdominal ultrasound
- Liver biopsy
- Liver enzymes (liver function tests)
- Liver MRI
- Serum alpha fetoprotein [9]

TYPES OF TREATMENT

Primary liver cancer is rarely detectable early, when it is most treatable. Secondary or metastatic liver cancer is hard to treat because it has already spread. The liver's complex network of blood vessels and bile ducts makes surgery difficult. Most treatment concentrates on making patients feel better and perhaps live longer.

- Patients with early-stage tumors that can be removed surgically have the best chance of long-term survival.
- In some patients, chemotherapy is given directly into the liver (chemoembolization) to reduce tumors to a size that may make surgery possible.
- Cryotherapy, or freezing the tumor, and radiofrequency ablation (RFA), using radio waves to destroy the tumor, may be used to treat some cases of liver cancer
- A liver transplant may be an option for those with both liver cancer and cirrhosis. Although this procedure is risky, it offers some chance of long-term survival. [10]

PREVENTION

- Preventing and treating viral hepatitis may help reduce your risk. Childhood vaccination against hepatitis B may reduce the risk of liver cancer in the future.
- Do not drink excessive amounts of alcohol.
- People with certain types of hemochromatosis (iron overload) may need to be screened for liver cancer.
- People who have hepatitis B or C or cirrhosis may be recommended for liver cancer screening. [11]

EXPERIMENTAL MODELS FOR HCC

Several rodent models have been used in defining the pathogenesis of HCC and have contributed to the current knowledge of HCC. Because of the physiologic and genetic similarities between rodents and humans, the short lifespan and the breeding capacity, rodents are often used for cancer research. Many chemically induced experiments have been conducted on rats (Rattus norvegicus) but Mice (Mus musculus) are also a favourite model for cancer because of the availability of gene targeting methods and the possibility of xenograft implantation. A Broad range of models are available which mainly includes

i. Chemically induced models
ii. Transgenic models
iii. Xenograft models

CHEMICALLY INDUCED MODELS

A few of the chemical compounds has been found to be carcinogens when administered in sufficient dose and given particular time span. They either belong to any of the two classes by the way which it induces tumor formation.

i. Genotoxic compounds - which are capable of inducing structural DNA changes, and
ii. Promoting compounds - which lack direct genotoxic capability, but enhance tumour formation after initiation by a hepatotoxic compound.

Chemical models of hepatocarcinogenesis often involve initiation by a carcinogen followed by a growth stimulus promoter to induce clonal expansion of initiated cells or by repeated administration of the carcinogen for a prlonged period of time.

DIETHYLNITROSAMINE INDUCED HCC

N-nitrosodiethylamine (DEN) is often used as a carcinogenic reagent. The carcinogenic capacity of DEN is situated in its capability of alkylating DNA structures. In the first step, DEN is hydroxylated to a-hydroxylnitrosamine. This bioactivation step

is oxygen-and NADPH-dependent and is mediated by cytochrome P450, an enzyme which has its highest activity in the centrilobular hepatocytes. After cleavage of acetaldehyde, an electrophilic ethyldiazonium ion is formed. This ethyldiazonium ion causes DNA damage by reacting with nucleophiles such as DNA-bases. Furthermore, oxidative stress caused by DEN can contribute to hepatocarcinogenesis. DEN works in a dose dependent manner, a single low initiation dose does not lead to the formation of neoplasm; administration of a high dose induces HCC after a period of latency. The development of HCC after administration of DEN not only depends on the dose but also on several other factors such as age, sex and strain of the animal. The yonger animals are found to develop HCC faster due to its high hepatocyte proliferation rate. Male rats are found to show 100% development while female rats show only 30% of tumor development. This gender related difference is due to the inhibitory effect of estrogen and stimulating effect of androgens on hepatocarcinogenesis. The tumor sensitive rats are found to develop HCC fater than tumor resistant rat strains. [12]

INFLAMMATION [13]

Inflammation is defined as the local response of living mammalian tissues to injury due to any foreign agent, in order to eliminate or limit the spread of injurious agent, followed by removal of the necrosed cells and tissues.

The agents causing inflammation are:

a) *Infective agents* like bacteria, viruses and their toxins, parasites, fungi.

b) *Immunological agents* like cell-mediated and antigen antibody reactions.

c) *Physical agents* like heat, cold, radiation, mechanical trauma.

d) *Chemical agents* like organic and inorganic poisons.

e) *Inert materials* such as foreign bodies

AGENT CAUSING INFLAMMATION [14, 15]

- Infectious agent : Bacteria, viruses and their toxins, fungi.
- Immunological agent : Cell mediated and antigen anti body reaction
- Chemical agents : Organic and inorganic poisons
- Inert materials : Foreign bodies

SIGNS OF INFLAMMATION

The inflammation was first described by Celsus who identified cardinal signs of inflammation as:

- Rubor (Redness)
- Tumor(Swelling)
- Calor (Heat)
- Dolor (Pain)
- Function Laesa(Loss Of Function)

Redness of inflammation is due to dilation of vascular bloods in injured area and heat is due to increased blood flow. Swelling occurs due to edema formation caused by fluid accumulation and plasma protein in the extra vascular spaces. Pain inflammation due to increased pressure in the tissue which leads to increased firing of pain afferents in affected area.

MEDIATORS OF INFLAMMATION

- Histamine
- Prostaglandins
- Leukotrienes
- Serotonin
- Lysosome
- Platelet activation factors
- Nitric oxide
- Cytokines
- Bradykinins

PHASES OF INFLAMMATION [16]

- Vasodilatation: Vasodilatation is the first phase of inflammation, caused by increase in vascular permeability result in exudation of fluid from blood into interstitial space
- Exudation: Exudation is the second phase of inflammation; it involves the filtration of leukocytes from blood into tissue

- Emigration of cells: It is the third phase of inflammation; it involves granuloma migration and tissue repair.

TYPES OF INFLAMMATION

Based on the defense capacity of the host and duration of response, inflammation can be classified as acute and chronic

A. Acute inflammation is of short duration, enduring less than 2 weeks and represents the early body reaction, resolves quickly and is usually followed by healing.

The main features of acute inflammation are:

I. Accumulation of fluid and plasma at the affected site.

II. Intravascular activation of platelets.

III. Polymorphonuclear neutrophils as inflammatory cells

B. Chronic inflammation is of longer duration and occurs either after the causative agent of acute inflammation persists for a long time, or the stimulus is such that it induces chronic inflammation from the beginning. The characteristic feature of chronic inflammation is presence of chronic inflammatory cells such as lymphocytes, plasma cells and macrophages, granulation tissue formation, and in specific situations as granulomatous inflammation. [12]

CAUSES OF INFLAMMATION

- *Microbial infections***:** Microbes include viruses, bacteria, protozoa, fungi and various parasites.
- *Hypersensitivity reactions*
- *Physical agents, irritant and corrosive chemicals*: Physical trauma, ultraviolet or other ionizing radiation, burns or excessive cooling ('frostbite') may cause tissue damage leading to inflammation. Corrosive chemicals such as acids, alkalis, oxidizing agents are also inflammatory stimulus that can cause direct tissue damage.
- *Tissue necrosis*: Lack of oxygen or nutrients results into inadequate blood flow and it is a potent inflammatory stimulus that can cause the death of tissues.

ROLE OF INFLAMMATION

1) Physiological role

- Eliminate cause of inflammation and to minimalize tissue damage

- To stop spreading of the cause of inflammation

- To activate processes of regeneration and repair

- Without inflammation, the tissues are not capable of healing

2) Pathological role

- Excessive or long-lasting reaction leading to tissue damage

- Role in pathogenesis of many diseases [17]

GOLDNANOPARTICLES /GOLD COLLOID SOLUTION

Nanotechnology is used for controlling multiple medical processes with effective influence on medicine .Nanotechnology is the engineering of functional systems at the molecular scale which involves several interdisciplinary fields, such as medicine, electronics, and biomaterials. Nanoparticles offer alternative option in cancer therapy both as drug delivery carriers and as direct therapeutic agents for cancer cell inactivation.

Goldnanoparticles /Colloidal Gold nanoparticle suspensions have been used in medicine from the ancient era. The size of particle varies from 10 to 100nm. Colloidal gold nanoparticles emit either an intense red colour (for particles 25nm) or green (for particles 50nm). Particles less or more than that emit different colours. Gold nano particles occur as clusters of gold atoms up to 100nm in diameter. They have unique optical, electronic, and molecular-recognition properties they are the subject of substantial research, with applications in a wide variety of areas in the field of medicine. Colloidal gold suspensions are widely used in imaging techniques such as detection of cancer etc. due to their fluorescent properties. Colloidal gold nanoparticles (AuNPs) are utilized in many modern technological and medical applications because of its unique characteristic in interaction with visible light. Their optical and electronics characteristics gave the chance to be applied in electronic uses, for example, as sensory probes,

Bioimaging, agents for medical therapy, drug delivery, medical applications and electronic instrumentalists. They are used in therapeutic agent carriage due to its large surface area/volume ratio, allowing their surface to be coated with numerous types of molecules including therapeutics and targeting agents. [18]

Among the currently investigated metal-based chemotherapeutic agents, gold nanoparticles hold a promising future as these compound exhibit different oxidation states and lower toxicity compared to other metal-based drugs. Generally, Gold nanoparticles with oxidation states II and III have potential applications in the field of medicine, particularly as chemotherapeutic agents for various cancers.

CHARACTERISTICS OF GOLD NANOPARTICLES

- Gold nanoparticles are chemically inert
- These have greater biological compatibility
- Optical properties like plasmon resonance are fluorescence and chemiluminescence having better exhibited by gold nanoparticles
- Gold nanoparticles provide microscopic probes for the study of the cancer cell
- Gold nanoparticles accumulate in the cancerous cell and show the cytotoxic effect i.e. apoptosis or necrosis of the specific cell and cell specific receptor
- These have high stability due to the gold-sulphur bonds

BIOLOGICAL PROPERTIES OF GOLD NANOPARTICLE

- Most of the biological applications of gold nanoparticles use it as a passive agent, either as a probe for electron microscopy or as a vehicle to deliver biomolecules into cells
- gold nanoparticles can be used as active agents to interfere directly with the cellular processes and possess anti-angiogenic and anti-tumor properties
- Gold nanoparticles can also be fabricated as a multifunctional nanoplatform for various biomedical applications such as detection and diagnosis
- Utilized the multifunctional platform of AuNPs by conjugating an antibody to bind to epidermal growth factor receptors (EGFR).[19]

GOLD NANOPARTICLES AGAINST CANCER

Metal complexes have dominated the modern-day use of drugs in cancer. Gold nanoparticles have been used widely in the treatment of various malignant tumors as chemotherapeutic agents. [20] Gold compounds are potential antitumor agents their use has been limited due to their mandatory side effects in the modern-day treatment of cancer. Development of resistance to drugs has also minimized the use of gold compounds in the current treatment of cancer. These unresolved disadvantages potentiate the research on cancer in the quest to find alternatives for the treatment of cancer .Gold complexes in cancer were highlighted in the early 1970s and 1980s. Gold phosphine complexes were the first to investigate for their antitumor activity in gold complexes. Auranofin a gold I phosphine complex showed excellent antitumor property in mice bearing in P388 leukemia. [21]

GOLD NANOPARTICLES AGAINST ANTI INFLAMMATORY

Gold nanoparticles increase cutaneous wound healing in mouse through its anti-inflammatory and antioxidative effects. AuNPs have anti-inflammatory properties through their capability to hinder expression of NF-kappa B and consequent inflammatory reactions. Many researchers have studied the anti-inflammatory effect of various nanoparticles and the releasing of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin (IL)-6 have been studied previously. The cytotoxic effects of gold nanoparticles were evaluated in macrophages and the expression of the inflammatory cytokines IL-6, IL-10 and TNF-α were quantified. It was found that NPs are cytotoxic to macrophages and are able to elicit an inflammatory response. [22] Gold or silver nanoparticles conjugated with heparin exhibited anti-inflammatory properties without any significant effect on systemic hemostasis Chen et al. reported a reduction in TNFα and IL-6 mRNA levels when 21-nm gold nanoparticles were injected into mice, a result attributed to fat loss and inhibition of inflammatory effects. Administration of nanoparticles of MnO2 caused accumulation of Mn in brain, spinal cord and muscle tissues of rats, leading to an impact on pain sensation. Finally, nanoparticles have also presented antitumor properties, which is quite interesting from the viewpoint of formulating new drugs against cancer. [23]

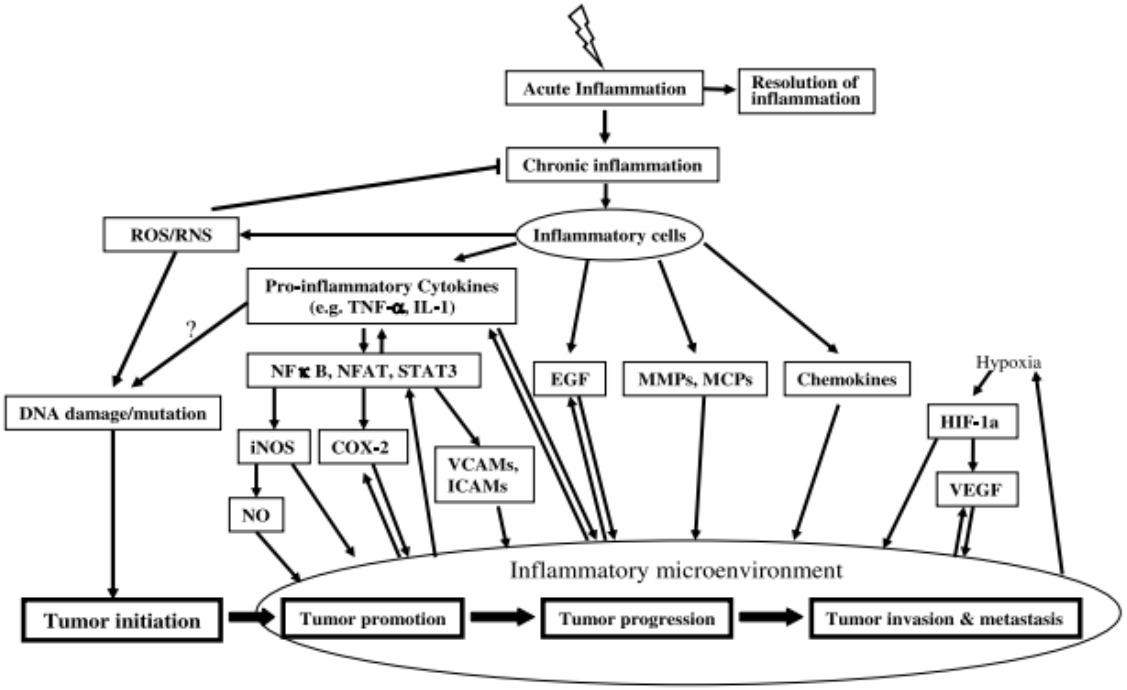


Figure: 2. Summary of mechanisms for the involvement of inflammation in cancer development. Tumor promotion indicates the process during which initiated cells develop into benign lesions. Tumor progression defines the process during which benign tumors progress to malignant carcinomas. [24]

APPLICATIONS OF GOLD NANOPARTICLES

A list of some of the applications of nano materials to biology or medicine is given below:

- Fluorescent biological labels
- Drug and gene delivery
- Bio detection of pathogens
- Detection of proteins
- Probing of DNA structure
- Tissue engineering
- Tumour destruction via heating (hyperthermia)
- Separation and purification of biological molecules and cells
- MRI contrast enhancement
- Phagokinetic studies [25]

2. REVIEW OF LITERATURE

Nikolaos m. dimitriou *et al.*, (2017) studied gold nanoparticles, radiations and the immune system: Current insights into the physical mechanisms and the biological interactions of this new alliance towards cancer therapy. Considering both cancer's serious impact on public health and the side effects of cancer treatments, strategies towards targeted cancer therapy have lately gained considerable interest. Employment of gold nanoparticles (GNPs), in combination with ionizing and non-ionizing radiations, has been shown to improve the effect of radiation treatment significantly. GNPs, as high-Z particles, possess the ability to absorb ionizing radiation and enhance the deposited dose within the targeted tumors. [26]

Sejal Patel *et al.*, (2016) revealed that annonaceous acetogenins are the major constituents of A. muricata. More than 100 annonaceous acetogenins have been isolated from leaves, barks, seeds, roots and fruits of A. muricata. It has wide potent anticancerous agents and it plays a key role towards many varieties of cancer, Acetogenins are potent inhibitors of NADH oxidase of the plasma membranes of cancer cells. These activities include anticancer, anticonvulsant, anti-arthritic, antiparasitic, antimalarial, hepatoprotective and antidiabetic, analgesic, hypotensive, antiinflammatory, and immune enhancing effects. The most promising activities are found to be its anticancer. [27]

Ana V. Coria-Te *et al.*, (2016) reviewed and focused on the phytochemicals contents, bioactivity, biological actions and toxicological aspects of extracts and isolated compounds, as well as medicinal uses of A. muricata had been identified in tropical regions to treat diverse ailments such as fever, pain, respiratory and skin illness, internal and external parasites, bacterial infections, hypertension, inflammation, diabetes and cancer. More than 200 chemical compounds has been identified and isolated from this plant the most important being alkaloids, phenols and acetogenins. Using in vitro studies, extracts and phytochemicals of A. muricata have been showed as an antimicrobial, anti-inflammatory, anti-protozoan, antioxidant, insecticide, larvicide, and cytotoxic to tumor cells. In vivo studies of the crude extracts and isolated compounds of A. muricata has

Showed to possess anxiolytic, anti-stress, anti-inflammatory, contraceptive, antitumoral, antiulceric, wound healing, hepato-protective, anti-icteric and hypoglycemic activities.[28]

Liu N *et al*., (2016) showed the extracts of Annona muricata used to cause apoptosis of various cancer cells in vitro, and inhibit tumor growth in vivo in animal models. Investigated the molecular mechanisms underlying liver cancer cell apoptosis triggered by the ethanol extract of leaves of Annona muricata .Liver cancer HepG2 cells were used as experimental model. MTT assay has employed and evaluated the cell viability. Showed that the extract was able to reduce viability and trigger apoptosis of the cancer cells. Proteomic analysis identified 14 proteins associated with the extract-elicited apoptosis, which included the increased expression levels of HSP70, GRP94 and DPI-related protein 5. Western blot analysis confirmed that the extract did up-regulated the protein levels of HSP70 and GRP94. Results from bio informatic annotation pulled out two molecular pathways for the extract notably, included endoplasmic reticulum (ER) stress. Results indicated that the ethanol extract of leaves of Annona muricata. Apoptosis of liver cancer cells through ER stress pathway, which supports the ethnomedicinal use of this herb as an alternative or complementary therapy for cancer. [29]

L.A. Dykman *et al*., (2016) studied Biomedical Applications of Multifunctional Gold-Based Nanocomposites andActive application of gold nanoparticles for various diagnostic and therapeutic purposes started in recent decades due to the emergence of new data on their unique optical and physicochemical properties. In addition to colloidal gold conjugates,growth in the number of publications devoted to the synthesis and application of multifunctional nanocomposites has occurred in recent years. This review considers the application in biomedicine of multifunctional nanoparticles that can be produced in three different ways. The first method involves design of composite nanostructures with various components intended for either diagnostic or therapeutic functions. [30]

Khwaja Salahuddin Siddiqi *et al*., (2016) Observed Recent advances in plant-mediated engineered gold nanoparticles and their application in biological system. For biosynthesis of gold nanoparticles different parts of a plant are used as they contain

Metabolites such as alkaloids, flavonoids, phenols, terpenoids, alcohols, sugars and proteins which act as reducing agents to produce nanoparticles. They also act as capping agent and stabilizer for them. They are used in medicine, agriculture and many other technologies. The attention is therefore focussed on all plant species which have either aroma or colour in their leaves, flowers or roots for the synthesis of nanoparticles because they all contain such chemicals which reduce the metal ions to metal nanoparticles. [31]

Ferdousi Akter *et al*., (2016) evaluated pharmacological and toxicological studies of an ayurvedic medicine Rasaraj Ras on biological system of the male Sprague-Dawley rats. In this study, the pharmacological and toxicological effects alongwith possible side effects of the classical ayurvedic formulation Rasaraj Ras (RR) which is used as a traditional medicine in the treatment of hemiplegia in the rural population were evaluated. All throughout the experimental period the RR treated animals were always maintaining negligible changes in body weight, but all throughout the experimental period no statistically significant increase or decrease was noted. There is a statistically significant decrease in the relative percent weight of the male rat heart. There is a statistically highly significant decrease in the absolute weight of the male rat liver. [32]

Micah D. K. Glasgow *et al*., (2016) studied Recent Developments in Active Tumor Targeted Multifunctional Nanoparticles for Combination Chemotherapy in Cancer Treatment and Imaging. Nanotechnology and combination therapy are two major fields that show great promise in the treatment of cancer. The delivery of drugs via nanoparticles helps to improve drug's therapeutic effectiveness while reducing adverse side effects associated with high dosage by improving their pharmacokinetics. Taking advantage of molecular markers over-expressing on tumor tissues compared to normal cells, an "active" molecular marker targeted approach would be beneficial for cancer therapy. These actively targeted nanoparticles would increase drug concentration at the tumor site, improving efficacy while further reducing chemo-resistance. [33]

Rosa Raybaudi-Massilia *et al*., (2015) evaluated the cytotoxic activity of aqueous and alcoholic extracts from Annona muricata L(soursop) seed and pulp on human tumor cell

lines of breast, prostate and cervix; as well as the antioxidant and antimicrobial properties of those extracts. The methanolic extract of soursop seed was obtained by two methods: Soxhlet apparatus and maceration. Human tumor cell lines from breast (MCF-7 and SKBr3), prostate (PC3) and cervix (HeLa), and fibroblasts (as control) were determined by the cytotoxic activity by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay.Antioxidant and antimicrobial activity were determined by the DPPH (1,1-diphenyl-2-picrylhydrazyl) and disc diffusion method respectively. This research suggested that consumption of soursop fruit will be a good alternative to prevent illness such as cancer of prostate and cervix. [34]

Shamshad Alam *et al*., (2015) suggested that the plant-based dietary supplements can reduce the risk of liver cancer. Nexrutine(NX), an herbal extract from Phellodendronamurense, has been shown the anti-inflammatory, anti-microbial and anti-tumor activities. The study has shown an anti-tumor potential of NX against Solt-Farber model with elimination of PH, rat liver tumor induced by diethylnitrosoamine (DEN) as carcinogen and 2-acetylaminofluorene (2-AAF) as co-carcinogen. The elucidation of mechanistic path-ways has explored in human liver cancer cells. Dietary intake has significantly decreased the cell proliferation and inflammation, as well as increased apoptosis in the liver sections of DEN/2-AAF-treated rats. Moreover, NX significantly decreased the viability of liver cancer cells and modulated the levels of Bax and Bcl-2 proteins levels. NX treatment resulted there is an increase in cytochrome-c release and cleavage of caspases 3 and 9. In addition, NX decreased the expression of CDK2, CDK4 and associated cyclins E1 and D1, while up-regulated the expression of p21, p27 and p53 expression. NX also enhanced phos-phorylation of the mitogen-activated protein kinases (MAPKs) ERK1/2, p38 and JNK1/2.Collectively, that these study suggested that NX-mediated protection against DEN/2-AAF-induced liver tumorogenesis involves decrease in cell proliferation and enhancement in apoptotic cell death of liver cancer cells. [35]

Olaoluwa Oyedeji *et al*., (2015) showed the biocidal activities of A. muricata leaf and the extracts were evaluated against 10 clinical strains of bacteria and 6 fungal strains. The ethyl acetate and n-butanol fractions of the leaf extract exhibited the highest antibacterial effects and indicated that greater quantities of the inherent bioactive agents

are extracted by these solvents. The extract showes broad spectrum antimicrobial effects which compared favorably with those of reference drugs – streptomycin and amphotericin B. Moreover, the n-butanol could be a better solvent for the extraction of the antifungal compounds from A. muricata leaf. This study has demonstrated appreciable biocidal activities of A. muricata leaf extracts which justify the ethnomedicinal use of the plant. [36]

Jyothy k bhaskaran *et al*., (2015) evaluate the effect of swarna bhasma on memory and learning in swiss albino mice. The study was conducted to evaluate the effect of swarna bhasma on memory and learning against hyoscine/ scopolamine induced amnesia in albino mice. Administration of gold in children is a popular practice in ayurveda. it is stated that pure gold if administered along with honey and ghee for a period of 6 months will enable the infant to remember things which are just heard. The trial drug showed significant decrease in transfer latency ($p<0.05$) both on 2nd and 3rd day of the study when compared with initial values and the control group. Swarna bhasma exhibited significant therapeutic effects on memory and learning in albino mice. [37]

Neha Mohan. P. V *et al*., (2015) evaluated anti-inflammatory activity in ethanolic extract of Coriandrum sativum L. using carrageenan induced paw oedema in albino rats. The present study investigates the anti-inflammatory activity in ethanolic extract of Coriandrum sativum. L using carrageenan induced paw edema in albino rats.The medicinal values of the Coriandrum sativum. L has been mentioned ancient literature as useful in disorders of inflammation.Dried leaves of Coriandrum sativum,powdered and extracted with ethanol using shaker. The anti-inflammatory was done by carrageenan induced hind paw edema method using plethysmometer. Indomethacin used as a standard drug. [38]

Balan Rajan *et al*., (2015) observed Carvacrol attenuates N-nitrosodiethylamine induced liver injury in experimental Wistar rats. Carvacrol is a main constituent in the essential oils of countless aromatic plants including Origanum Vulgare and Thymus vulgari, which has been assessed for substantial pharmacological properties. In recent years, notable research has been embarked on to establish the biological actions of

Carvacrol for its promising use in clinical applications. The present study is an attempt to reveal the protective role of Carvacrol against N-Nitrosodiethylamine (DEN) induced hepatic injury in male wistar albino rats. DEN is an egregious toxin, present in numerous environmental factors, which enhances chemical driven liver damage by inducing oxidative stress and cellular injury. [39]

Sherien k hassan *et al.*, (2014) therapeutic and chemopreventive effects of nano curcumin against diethylnitrosamine induced hepatocellular carcinoma in rats. The present study is designed to investigate the preventive and therapeutic effects of nano curcumin (Nano Cur) against diethylnitrosamine (DEN) induced hepatocellular carcinoma (HCC) in rats. Administration of DEN to rats in group II significantly increased relative liver weight, serum liver function enzymes, serum sialic acid, vascular endothelial growth factor and hepatic thiobarbituric acid reactive substances this was accompanied by significant decrease in serum albumin and tissue antioxidants (GPx, GST, SOD, CAT and GSH). [40]

B. Arirudran *et al.*, (2014) alteration in levels of minerals in den induced hepatocellular carcinoma in wistar albino rats. The present attempt has been made to evaluate, and examine the levels of minerals in serum and liver in DEN induced hepatocellular carcinoma in wistar albino rats for possible chemopreventive effect. In hepatocellular carcinogenesis complications such as hepatic fibrosis and cirrhosis may lead to several abnormalities in mineral metabolism, hence attempt is made to evaluate on the level of minerals. Hepatic cancer was induced by single dose of intraperitoneal injections of DEN (200mg/kg body weight) followed by phenobarbital of 0.05% mixed with drinking water for 20 weeks. Concentration of calcium, magnesium, sodium and potassium were assessed in the serum and liver at the end of experimental period. [41]

Jihyoun Lee *et al.*, (2014) Evaluated Gold nanoparticles in breast cancer treatment: Promise and potential pitfalls. Despite remarkable achievements in the treatment of breast cancer, some obstacles still remain. Gold nanoparticles may prove valuable in addressing these problems owing to their unique characteristics, including their enhanced permeability and retention in tumor tissue, their light absorbance and surface plasmon

resonance in near-infrared light, their interaction with radiation to generate secondary electrons, and their ability to be conjugated with drugs or other agents. [42]

Rajesh kumar soni *et al*., (2014) Observed anti-inflammatory activity of kirganelia reticulata (poir). baill. root by carrageenan-induced rat paw oedema model. to evaluate anti-inflammatory activity of ethanolic roots extract of kirganelia reticulata (poir). baill. by carrageenan- induced rat paw oedema . ethanolic roots extract of k. reticulata was investigated for anti-inflammatory activity by carrageenan induced right hind rat paw oedema in wistar rats at the dose of 200 and 300mg/kg, p. o. (per orally). The ethanolic extract of k. reticulata root shows significant anti-inflammatory activity ($p<0.05$ and $p<0.01$) at the dose of 300mg/kg, p.o. $p < 0.001$ .when compared to control. The results obtained demonstrated that ethanolic roots extract of k. reticulata (poir) baill. has potential health benefits as it showed dose dependant anti-inflammatory activity. [43]

Khetbadei Lysinia Hynniewta Hadem *et al*., (2014) Observed Inhibitory potential of methanolic extracts of Aristolochia tagala and Curcuma caesia on hepatocellular carcinoma induced by diethylnitrosamine in BALB/c mice.This study was to evaluate the anti-carcinogenic properties of the crude methanolic extracts of roots of AT and rhizomes of CC in BALB/c mice.Exposed to a hepatocarcinogen, diethylnitrosamine (DEN). To evaluate the effects of these two HPE either alone or following DEN exposure, serum transaminases (aspartate aminotransferase [AST], alanine aminotransferase [ALT]), alkaline phosphatase (ALP), and cancer marker enzyme acetylcholine esterase (AChE) were assayed in mice. [44]

Arun raj.R *et al*., (2014) evaluated the methanolic extract of Gordonia obtusa (Theaceae) for its effects on growth in MCF-7 breast cancer cell lines using MTT assay. These cell lines studied the extract decreased cell viability, inhibited cell proliferation, and induced cell death in a dose dependent manner. The present study demonstrated that methanolic leaf extract of Annona muricata showed more anti-cancer activity compared to its bark extract. The methanolic leaf and bark extracts of Annona muricata was tested for its antibacterial effect successfully showed zone of inhibition against the tested microorganism. The maximum zone of inhibition was shown by the methanolic leaf

extract of Annona muricata against Escherichia coli. So it could be a reliable source of potent pharmacophore for treatment of disease like cancer. [45]

Mitsuru Futakuchi *et al.*, (2013) observed that N-nitrosomorpholine (NMOR) given after a multi-carcinogenic treatment induced liver carcinomas with 56% lung metastasis. An additional treatment with diethylnitrosamine (DEN) with NMOR further enhanced the incidence of hepatocellular carcinoma (HCC) with lung metastasis. It is further revised the duration of NMOR treatment to establish an animal model with a simple experimental protocol and mechanisms of HCC metastasis and development of anti-metastatic therapeutics. Observed that the DEN exposure followed by 16-week treatment with NMOR to be a most efficient protocol for the induction of hepatocellular metastasizing to the lung. [46]

Attalla f. el-kott *et al.*, (2013) studied the histopathological, immunohisto chemical and ultrastructural alterations following administration of nigella sativa in rats hepatocellular carcinoma. Hepatocellular carcinoma (HCC) is the 3rd greatest cause of carcinoma-related deaths. In the present study, the Nigella sativa was used as a pharmaceutical agent in the hepatocarcinogenesis of rats which induced by Diethylnitrosamine (DEN). Four groups of animals were used and fed ad libitum. The 1st group was a control; the 2nd was fed ad libitum and given 0.2 g/rat/day orally in a watery suspension till the time of sacrifice. The 3rd group was intraperitoneally injected with a single dose of DEN 150 mg k-1 b.wt. The 4th group was intraperitoneally injected with DEN and after one week, the each rat was given Nigella sativa as in group 2 at dose 0.2 g/rat/day. [47]

Sohail Akhter *et al.*, (2012) evaluated Gold nanoparticles in theranostic oncology: current state-of-the-art and observed the recent progress in the field of gold nanomaterials in cancer therapy and diagnosis. Moreover, concern about the toxicity of gold nanomaterials is addressed. Gold nanoparticles (GNPs) have gained much attention as platform for drug delivery and diagnostic applications because of their unique surface characteristics, allowing easy functionalization with chemicals that alter their circulation behavior or redirect them to target cells. [48]

Magda Ismail Youssef *et al*., (2012) studied Expression of Ki 67 in hepatocellular carcinoma induced by diethylnitrosamine in mice and its correlation with histopathological alterations. Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide and the prognosis still remains dismal, so the present work was planned to assess the prognostic value of Ki67 in mice model of HCC induced by diethylnitrosamine (DEN), in addition to its correlation to the histopathological changes. histopathological study revealed spotty necrosis with enlarged nuclei and cholestasis 6 weeks after DEN injection. [49]

Anil Kumar *et al*., (2012) observed the Gold nanoparticles functionalized with therapeutic and targeted peptides for cancer treatment. Functionalization of nanostructures such as gold nanoparticles (AuNPs) with different biological molecules has many applications in biomedical imaging, clinical diagnosis and therapy. Researchers mostly employed AuNPs larger than 10 nm for different biological and medicinal applications in previous studies. Herein, we synthesized a novel small (2 nm) AuNPs, which were functionalized with the therapeutic peptide, PMI (p12), and a targeted peptide, CRGDK for selective binding to neuropilin-1(Nrp-1) receptors which overexpressed on the cancer cells and regulated the process of membrane receptor mediated internalization. [50]

Srisesharam srigopalram *et al*., (2012) studied the effect of terminalia chebula retz on den induced hepatocellular carcinogenesis in experimental rats. hepatocellularcarcinoma (HCC), a highly aggressive form of solid tumor, has been increasing in south east asia. The lack of effective therapy necessitates the introduction of novel chemo preventive strategies to counter the mortality associated with the disease. Towards this goal, the present study evaluates the chemo preventive potential of t.chebula aqueous extract (tce) by estimating the levels of lipid peroxidation and assaying activities of various marker enzymes in diethylnitrosamine (den) induced liver cancer bearing rats. the daily oral treatment of tce (50 mg/kg bwt) to liver cancer bearing rats demonstrated a significant ($p<0.05$) decline in lipid peroxidation, pathophysiological marker enzyme (ast, alt, alp, ldh, γ-gt and 5'nt) levels and increase in enzymic antioxidants (sod, cat, gpx, gr and gst) status. [51]

Erik C Dreaden *et al*., (2012) Studied size matters: gold nanoparticles in targeted cancer drug delivery. Cancer is the current leading cause of death worldwide, responsible for approximately one quarter of all deaths in the USA and UK. Nanotechnologies provide tremendous opportunities for multimodal, site-specific drug delivery to these disease sites and Au nanoparticles further offer a particularly unique set of physical, chemical and photonic properties with which to do so. This review will highlight some recent advances, by our laboratory and others, in the use of Au nanoparticles for systemic drug delivery to these malignancies and will also provide insights into their rational design, synthesis, physiological properties and clinical/preclinical applications, as well as strategies and challenges toward the clinical implementation of these constructs moving forward. [52]

K. Balamurugan *et al*., (2012) evaluation of Luteolin in the Prevention of N-nitrosodiethylamine-induced Hepatocellular Carcinoma Using Animal Model System. Hepatocellular carcinoma (HCC) is one of the commonest tumors worldwide. The treatment of HCC is vital for disease diagnosis and prognosis, as the liver is the most important organ controlling metabolic functions. Now-adays, western folklore medicines are largely dependent on the phyto compounds which are highly effective in therapy and with low side effects. Luteolin is a flavonoid (3,4,5,7-Tetrahydro flavones) possess anti-inflammatory, anticancer and anti-allergic property. [53]

Sasika d. bhalke *et al*., (2012) studied anti- inflammatory and anti nocicep tive activity of pterospermum acerifolium leaves. pterospermum acerifolium willd (family: sterculiaceae) has long been used traditionally for the treatment of painful inflammatory conditions in the indian folk medicine. in the present study we have evaluated antinociceptive effects and anti-inflammatory effects of unsaponified petroleum ether extract of pterospermum acerifolium leaves (uspel, 100 and 200 mg/kg orally) and isolated β-sitosterol (10 and 20 mg/kg) from the leaves. uspel and β-sitosterol are evaluated for its anti-inflammatory activity in carrageenan-induced paw edema model in rats and analgesic activity in acetic acid-induced writhing, hot plate and formalin induced paw licking models in mice. [54]

Zhao-Zhin Joanna LIM *et al*., (2011) studied the Gold nanoparticles in cancer therapy. The rapid advancement of nanotechnology in recent years has fuelled a burgeoning interest in the field of nanoparticle research, in particular, its application in the medical arena. A constantly expanding knowledge based on a better understanding of the properties of gold nanoparticles (AuNPs) coupled with relentless experimentation means that the frontiers of nanotechnology are constantly being challenged. At present, there seems to be heightened interest in the application of AuNPs to the management of cancer, encompassing diagnosis, monitoring and treatment of the disease. [55]

Naina Mohamed Pakkir Maideen *et al*., (2011) evaluated the chemopreventive effect of methanol extract of Phyllanthus polyphyllus (MPP) against N-nitrosodiethylamine (DEN) induced liver Phenobarbital promoted tumor. These agents significantly reduces tumor incidence, delay tumor onset and also have minimal long-term toxicity, The present study was undertaken to establish the cancer chemopreventive efficacy of MPP against DEN induced malignancy of liver.The extract phyllanthus polyphyllus shows result for hepatocarcinoma. [56]

C. Lasagna-Reeves *et al*., (2010) performed the Bioaccumulation and toxicity of gold nanoparticles after repeated administration in mice. Gold nanoparticles (GNPs) offer a great promise in biomedicine. Currently, there is no data available regarding the accumulation of nanoparticles in vivo after repeated administration. The purpose of the present study was to evaluate the bioaccumulation and toxic effects of different doses (40, 200, and 400 μg/kg/day) of 12.5 nm GNPs upon intraperitoneal administration in mice every day for 8 days. The gold levels in blood did not increase with the dose administered, whereas in all the organs examined there was a proportional increase on gold, indicating efficient tissue uptake. [57]

Philippa Newell *et al*., (2008) carried out experimental models for hepatocellular carcinoma by using mouse (mus musculus) as experimental animal and it has considered the best model systems for cancer because of the availability of gene targeting methods. They have used some of the technical methods for evaluation such like: 1.xenograft: Orthotopic xenograft: model in which hepatoma 129 cells originating from C3H mice are

injected into fibrotic livers of mice pretreated with TAA and EtOH, 2.Transgenic method: inducible transgenic: Tet-inducible Met expression under albumin promoter: 60% HCC at 12 months; tumors regressed when transgene (Tg) has been inactivated 3.endogenous GEM: conditional gene targeting: Cre-mediated liver specific PTEN-/- knockout: 66% HCC at 8 months. They had described both traditional models of carcinogenesis had an expression of oncogenes and tumor suppressor genes in genetically altered to produce HCC, and in other models tumor formation occurs dependent on inflammation, hence these (mus musculus) mice may show good response for animal while hepatocellular carcinoma screening is done. [58]

Nermin A. H. Sadik *et al.*, (2008) designed to study the efficacy of dietary supplementation with blueberries (BB) on diethylnitrosamine (DEN)-initiated hepatocarcinogenesis in male wistar rats. The results showed that BB caused significant decrease in the elevated serum levels of alpha-fetoprotein (AFP), homocysteine (Hcy) along with levels of glutathione (GSH), deoxyribonucleic acid (DNA), ribonucleic acid (RNA)and activity of Glutathione reductase (GR) in liver. Normalization of elevated 2-macroglobulin (2M) and total antioxidant capacity (TAC) levels in serum, hepatic glutathione-S-transferase (GST), glutathione peroxidase (GPx) activities and liver weight was achieved whereas body weight was significantly decreased. Moreover, no significant change was observed in elevated relative liver weight, hepatic glucose-6-P-dehydrogenase (G6PD), lactate dehydrogenase (LDH) along with serum amino transferases, alkaline phosphatase (ALP) and glutamyltransferase (GT) activities. Significant increase in reduced hepatic activity of xanthine oxidase (XO) was achieved and histopathological damage was minimized in BB-treated group. It is suggested that BB suppress DEN- induced hepatocarcinogenesis and could be developed as a promising chemopreventive natural supplement for liver cancer. [59]

Sonia bajaj *et al.*, (2000) studied anti-cataleptic, anti-anxiety and anti-depressant activity of gold preparations used in indian systems of medicine. Traditional gold preparations used in Ayurveda and Unani-Tibb exhibited anxiolytic, antidepressant and anti-cataleptic actions with wide margin of safety. Anxiety, depression and mental health problems in general and senile neurological disorders in particular are widely prevalent

in modern fast-paced life with a multitude of stressful conditions While gold is used only for the treatment of rheumatoid arthritis in modern medicine 7-9 it is attributed with varied medicinal properties in Ayurveda and Unani-Tibb including nervine tonic effects and utility in neuropsychiatric. [60]

Ranjan basak *et al* ., (2000) evaluated inhibition of Diethylnitrosamine induced rat liver chromosomal and the DNA-strand breaks by synergistic supplementation of vanadium and 1K,25-dihydroxyvitamin D3.These drug has inhibited growth and induced differentiation of a variety of cell types. Synergistic of both V and 1, 25(OH)2D3 DEN injection has found to offer significant protection against generation of single-strand breaks. The study has result of elevation of the mitotic rate in the Liver. Therefore, it seems possible that the elevation of the mitotic rate during DEN treatment favored production and increase in the number of cells with abnormal chromosomes, and the drug (vanadium and 1K, 25-dihydroxyvitamin D3) shows an anti hepatocarcinogenesis activity. [61]

3. AIM & OBJECTIVE

AIM

The aim of the present study was to evaluate the pharmacological action of Gold nanoparticles.

OBJECTIVE

The objectives of the present study include:

- Evaluation of *in vitro* anti-cancer activity
- Evaluation of *in vivo* anti-inflammatory activity
- Evaluation of *in vivo* anti-cancer activity

4. PLAN OF WORK

- Review of literature
- Qualitative analysis (Particle size, Surface property, Elemental composition)
- Acute toxicity studies
- Evaluation of anti-cancer study
    1. DEN induced liver cancer (in vivo)
    2. MTT assay (in vitro)
- Evaluation of anti-inflammatory activity
    1. Carrageenan induced paw oedema
    2. Histamine induced paw oedema
    3. Serotonin induced paw oedema
- Parameters to be evaluated

  Estimation of serum biochemical parameters
    1. SGOT
    2. SGPT
    3. Alkaline phosphate (ALP)
    4. Total protein

  Estimation of Haematological parameters
    1. Red blood cells (RBC)
    2. White blood cells (WBC)

  Physical parameters
    1. Body weight
    2. Liver weight
- Histopathological studies
- Statistical analysis

5. MATERIALS AND METHODS

5.1. Collection of the Formulation

Thanga parpam was procured from Jaya indian medicine pharmaceutical PVT.LTD (Chennai) [62]

5.1.1. Uses

- Neuromuscular disorders
- Respiratory disorder
- Tremors(shake)
- Supportive therapy in cancer
- Arthritis
- Low sperm count

5.2. QUALITATIVE ANALYSIS (PARTICLE SIZE, SURFACE PROPERTY, ELEMENTAL COMPOSITION)

5.2.1. Synthesis and Characterization.

Gold colloids were synthesized by reduction of chloroauric acid with sodium citrate using the Turkevich method. [63]

The elemental composition of gold colloids was measured by ICP-MS (inductively Coupled Plasma Mass Spectroscopy) 7700x Agilent and Energy Dispersive Spectroscopy (EDS attached to SEM Hitachi S 3400N. The gold nanoparticle (Jaya) was suspended in DI water and deposited on pre-cleaned glass slides for SEM measurements. Dynamic Light Scattering (DLS) measurements were done by using a Nicomb 380 instrument. Elemental composition was measured by EDS-SEM and ICP-MS after digestion with aqua regia.

5.2.2. Turkevich method

- The method pioneered by J. Turkevich et al. in 1951 and refined by G. Frens in the 1970s, is the simplest one available. In general, it is used to produce modestly monodisperse spherical gold nanoparticles suspended in water of

around 10–20 nm in diameter. Larger particles can be produced, but this comes at the cost of monodispersity and shape. It involves the reaction of small amounts of hot chloroauric acid with small amounts of sodium citrate solution. The colloidal gold will form because the citrate ions act as both a reducing agent and a capping agent.

- Recently, the evolution of the spherical gold nanoparticles in the Turkevich reaction has been elucidated. It is interesting to note that extensive networks of gold nanowires are formed as a transient intermediate. These gold nanowires are responsible for the dark appearance of the reaction solution before it turns ruby-red.
- To produce larger particles, less sodium citrate should be added (possibly down to 0.05%, after which there simply would not be enough to reduce all the gold). The reduction in the amount of sodium citrate will reduce the amount of the citrate ions available for stabilizing the particles, and this will cause the small particles to aggregate into bigger ones (until the total surface area of all particles becomes small enough to be covered by the existing citrate ions).

5.2.3. Dynamic light scattering (DLS)

- Dynamic light scattering (DLS) is a technique in physics that can be used to determine the size distribution profile of small particles in suspension or polymers in solution.

- In the scope of DLS, temporal fluctuations are usually analyzed by means of the intensity or photon auto-correlation function (also known as photon correlation spectroscopy or quasi-elastic light scattering).

- In the time domain analysis, the autocorrelation function (ACF) usually decays starting from zero delay time, and faster dynamics due to smaller particles lead to faster decorrelation of scattered intensity trace. It has been shown that the intensity ACF is the Fourier transformation of the power Spectrum, and therefore the DLS measurements can be equally well performed in the spectral domain.

- DLS can also be used to probe the behavior of complex fluids such as concentrated polymer solutions.

- DLS is used to characterize size of various particles including proteins, polymers, micelles, carbohydrates, and nanoparticles. If the system is not dispersing in size, the mean effective diameter of the particles can be determined.
- Dynamic light scattering provides insight into the dynamic properties of soft materials by measuring single scattering events, meaning that each detected photon has been scattered by the sample exactly once.

5.2.4. Field Emission Scanning Electron Microscopy (FESEM)

To study the surface structure of the Gold nanoparticles and Gold colloid solution FESEM analysis was carried out on FEI Nova NanoSEM 450. Resolution: 1.0 nm at 15kV, 1.4 nm at 1kV and 1.8 nm at 3kV and 30Pa. Software used was xT microscope control.

5.2.5. X-Ray Diffraction (XRD)

- XRD measurements were carried out in symmetric reflection mode with a custom-built diffract meter equipped with pyrolytic graphite monochromator and analyzer crystals.
- The elemental composition of the Gold nanoparticle was confirmed used XRD analysis.
- The XRD pattern of Gold nanoparticle and Gold colloid solution sample reflects gold metal as the major phase along with certain impurities
- Cu K-alpha radiation = 0.15418 nm was used for the measurements

5.2.6. Energy Dispersive X-ray Spectrometry (EDS)

Elemental composition of the Gold nanoparticle and Gold collid solutions are determined by EDS analysis. It was carried out on Bruker X Flash 6I30. Resolution: 123eVat Mn k-alpha and 45eV at C K-alpha and element detection range from 4 Be to 95 Am. Software used was Espirit 1.9. [63]

5.3. *IN VITRO* ANTICANCER ACTIVITY

5.3.1. MTT assay

3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells.

After 48 h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37^0C for 4h. The medium with MTT was then discarded and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The percentage cell viability was then calculated with respect to control as follows

% Cell viability = [A] Test / [A]control x 100

5.3.2. Cell line

The human breast adenocarcinoma cell line (MCF7) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). The cells were maintained at 370C, 5% CO2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

5.3.3. Cell treatment procedure

The monolayer cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted by tryphan blue exclusion using a hemocytometer. Dilutions were made with medium containing 5% FBS to give final density of 1x105 cells/ml. One hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 370C, 5% CO2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the test sample. The sample was initially dispersed in phosphate buffered saline and an aliquot of the sample solution was diluted to twice the desired

final maximum test concentration with serum free medium. Additional four serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulting in the required final sample concentrations. Following sample addition, the plates were incubated for an additional 48 h at 370C, 5% CO2, 95% air and 100% relative humidity. The medium containing without samples were served as control and triplicate was maintained for all concentrations. [65, 66]

5.4. PHARMACOLOGICAL EVALUATION OF THE GOLD NANOPARTICLES

Animals and Diet

Male Sprague Dawley rats weighing about 120-200gm were used for study. The animals were housed under controlled conditions of temperature (20-25^{0}C) and photoperiod of 12-h light/dark cycle.

All the rats were fed with pelletized commercial chow diet and fresh water *ad libitum* throughout the experimental period, and weight gain was measured in a weekly manner.

All animal procedures were performed after obtaining approval from the animal ethical committee and accordance with the recommendations for the proper care and use of laboratory animals by CPCSEA.

5.4.1. *INVIVO* ANTICANCER ACTIVITY

5.4.1.1. DEN Induced Hepatocellular carcinoma

Principle

- Diethylnitrosoamine (DEN), also known as N-nitrosodiethylamine, is widely used as a carcinogen in experimental animal models. Upon administration of DEN to the animal, either orally or as intraperitoneal injection, tumors will be induced in organs like, liver, gastrointestinal tract, skin, respiratory tract and hematopoietic cells.

- DEN is not a direct carcinogen and hence it needs to be biotranformed by cytochrome P450 (CYP) enzymes present in the liver. This biotransformation in DNA-adducts formation carries through an alkylation mechanism.
- CYP 2E1 has been considered to be important for DEN activation since CYP2E1-deficient animals show lower tumor incidence and multiplicity for DEN-induced hepatocarcinogenesis. This result firmly proposes that CYP2E1 plays an indispensable role in the activation of DEN, although several other CYP enzymes are proposed to catalyze DEN bioactivation in vivo. [67]

Procedure

5.4.1.2. Induction of Hepatocellular carcinoma

Thirty six male Sprague Dawley rats were taken and divided in to six groups and each group contains six animals. All animals were fasted overnight. DEN 200 mg/kg was injected intraperitonealy by dissolving in normal saline for the induction of hepatocarcinoma for the groups 2 to 6 from the day 0. After 1 week, group 1 received only normal saline. Group 2 received only DEN (200 mg/kg), group 3 received standard drug (5-fluro uracil) intraperitoneally, group 4 and 5 received the treatment with the GNP (30 mg/kg and 60 mg/kg respectively) orally and group 6 received the treatment with the Gold colloidal solution intraperitoneally. After treatment for six weeks, the changes in body weight of the animals were observed. Then the animals were fasted overnight and the next day, the blood was collected from the animal through retro orbital puncture for the determination of hematological parameters and serum was separated for the estimation of biochemical parameters. The rats were sacrificed by cervical dislocation and the liver was isolated and sent to laboratory for histopathological study. [68]

Table: 1 Pharmacological Model for DEN induced HCC

Groups	Sample Size	Group Specification
Group I	6	Normal (Normal saline)
Group II	6	DEN Control
Group III	6	DEN + Standard [5 Flurouracil (20mg/kg) body wt i.p)]
Group IV	6	DEN+GNP(Low dose-30mg/kg)
Group V	6	DEN + GNP (High dose-60mg/kg)
Group VI	6	DEN+GCS (60µg/100g)

5.4.2. ESTIMATION OF HAEMATOLOGICAL PARAMETERS

Blood Collection

After the end of the treatment period (90 days) of the animals were anaesthetized with ketamine (i.p) and its blood was collected by retro orbital puncture with addition of EDTA for the enumeration of blood cells i.e., RBC and WBC. The estimation of various biochemical parameters carried out by using blood sample without adding EDTA.

Separation of Serum

The serum was separated from the blood by centrifuging at 10,000 rpm for 10 minutes. The separated serum was collected and used for the estimation of haematological parameters. For the estimation of the biochemical parameters such as Alkaline phosphatase (ALP), Serum glutamate oxalo acetate transaminase (SGOT), Serum glutamate pyruvate transaminase (SGPT), Serum creatinine and total protein, Such as

5.4.3. Enumeration of red blood cells (RBC)

Requirements

- RBC diluting fluid (Hayem's fluid)
- Counting chamber (Neubauer's chamber)
- RBC pipette
- Microscope with 45 X lens

Procedure

RBC pipette was filled with blood up to the mark 0.5. RBC diluting fluid (Hayem's fluid) was filled up to the mark 101. The pipette was rolled between the palms to ensure thorough mixing of blood with diluting fluid and kept for some time. The counting chamber was placed and the RBC squares were focused under low power and after identification of the markings turn to high power. The first 3-4 drops of blood mixture was discarded and it was mixed once again. The counting chamber was charged with the mixed blood. After that mount the slide and allowed the fluid to settle down by using a 45 X lens. The RBC was counted in the corner and middle squares. The numbers of cells were expressed as 10^6 cells/mm3.

5.4.4. Enumeration of white blood cells (WBC)

Requirements

- WBC diluting fluid (Turk's fluid)
- Counting chamber (Neubauer's chamber)
- WBC pipette
- Microscope with 45 X lens

Procedure

WBC pipette was filled with blood up to the mark 0.5. RBC diluting fluid (Turk's fluid) was filled up to the mark 11. The pipette was rolled between the palms to ensure thorough mixing of blood with diluting fluid and kept for some time. The counting chamber was placed and the WBC squares were focused under low power and

after identification of the markings turn to high power. The first 3-4 drops of blood mixture was discarded and it was mixed once again. The counting chamber was charged with the mixed blood. After that mount the slide and allowed the fluid to settle down by using a 45 X lens. The WBC was counted in the corner and middle squares. The number of cells was expressed as 10^3 cells/mm3. [69]

5.4.5. Estimation of serum biochemical parameters

5.4.5.1. Estimation of Alkaline phosphatase (ALP)

Method

Kinetic photometric test-according to IFCC (International Federation of Clinical Chemistry and Laboratory Medicine)

Principle

ALP hydrolysis P-Nitrophenyl phosphate to P-Nitrophenol and phosphate.

Reaction

P-Nitrophenylphosphate+H2O ⟶ P-Nitrophenol + phosphate

Reagents

Composition of ALP reagent

Reagent	Composition	Quantity
R1	**Diethanolamine**	**1.5 Mol/L**
	Magnesiumchloride	0.6 m Mol/L
R2	p-Nitrophenyl PhosphateAnalogue	0.070 m Mol/L
	Sodium Azide 10%	

Procedure

800 µL of Reagent 1 was taken in a 5ml test tube and to it 200 µL of Reagent 2 was added and mixed. To it 25 µL of serum sample was added and mixed well after the sample was allowed to run. [70]

5.4.5.2. Serum Glutamate Oxaloacetate Transaminase (SGOT)

Principle

SGOT

L-aspartate+2-oxaloglutarate ⟶ L-Glutamate + oxaloacetate

LDH

Oxaloacetate+ $NADH+H^+$ ⟶ D-Malate+NAD^+

This modified formula for the assay of AST/GOT, as recommended by the IFCC (International federation of clinical chemistry). The IFCC reference method includes pyridoxal phosphate (PP) function as coenzyme in AA transfer, therefore addition of PP results in increased enzyme activity. It avoids falsely low activity in samples containing insufficient endogenous PP, e.g. from patients with myocardial infarction, liver disease and intensive care patients.

Method: Optimized UV test according to the IFCC (International Federation of Clinical Chemistry and laboratory medicine)

Composition of SGOT reagent

Reagents	Composition	Quantity
R1	Tris pH-7.8	80 m Mol/L
	Preservatives	
	Stabilizers	
R2	L-Aspartate	240 mMol/L
	NADH	0.18 mMol/L
	Pyridoxal 5 Phosphate	
	Lactate Dehydrogenase	≥900 U/L
	Malate Dehydrogenase	≥600 U/L
	Alpha Ketoglutarate	12 mmol/L

Procedure

800 µL of Reagent 1 was taken in a 5 ml test tube and to it 200 µL of Reagent 2 was added and mixed. To it 100 µL of serum sample was added, mixed well and the sample was allowed to run.

5.4.5.3. Serum Glutamate Pyruvate Transaminase (SGPT)

Principle

SGPT

$$\text{L-alanine+2-oxoglutarate} \longrightarrow \text{L -glutamate+pyruvate}$$

$$\text{Pyruvate+NADH+H}^{+} \longrightarrow \text{D-Lactate+NAD}$$

Addition of pyridoxal-5-phosphate (P-5-P) stabilizes the transaminases and avoids false low values in samples containing insufficient endogenous P-5-P, e.g. from patient with myocardial infarction, liver disease and intensive care patients.

Method

According to international federation of clinical chemistry and laboratory medicine (IFCC)

Reagents

Composition of SGPT reagent

Reagents	Composition	Quantity
R1	L- Alanine	≥200 U/L mMol
	LDH(lactate dehydrogenase)	≥1500 U/L
	2-Oxoglutarate	15 mMol/L
R2	NADH	1.05 mMol/L
	Pyridoxal-5-phosphate	
	Alpha-ketoglutarate	>35 mMol/L

Procedure

800µL of Reagent-1 was taken in a 5ml test tube and 200 µL of Reagent-2 was added and mixed. To it 100µL of serum sample was added and the sample was allowed to run.[71]

5.4.5.4. Estimation of Total Protein in Liver tissue

Principle

The method is based on the burette reaction, formation of a protein–copper complex and reduction of phosphour molybdenum tungstate reagent (Folin–Ciocalteu Phenol reagent) by tyrosine and tryptophan residues of protein to form a colored product. [72]

Reagents

Solution A: 1ml CuSO4 5.H2O (1%) + 1ml Sodium potassium tartarate (2%) + 98 ml 2% Na2CO3 in 0.1N NaOH.

Solution B: Folin Ciocalteu reagent and distilled water was mixed in 1:1 ratio just before use.

Procedure

The animals were sacrificed after treatment by euthanasia. Liver was isolated and washed with normal saline. The isolated liver was homogenized in 0.01 M Tris HCL. 0.01 ml of tissue homogenate (30%) was diluted to 1.2 ml and mixed with 6ml of solution A. The mixture was incubated at room temperature for 10 min and added 0.3 ml solution B. Mixed well immediately and kept at room temperature for 30 min. Optical density was measured at 750 nm. The amount of protein was calculated from the standard curve of Bovine Serum Albumin.

5.4.6. HISTOPATHOLOGICAL STUDIES

The liver from all groups were removed rapidly, and thoroughly rinsed with ice-cold saline. After 24 h of fixation followed by embedding in a paraffin block, it was cut into sections of 5 micron onto a glass slide and stained with hematoxylin-eosin for histological assessment of the tissue. Sections of liver were examined by light microscope at 10 and 40 X magnification.

5.4.7. SCREENING OF ANTI INFLAMMATORY ACTIVITY

Experimental methods:

Anti-inflammatory activity was assessed by carrageenan, histamine and Serotonin induced rat paw oedema methods

5.4.7.1. CARRAGEENAN INDUCED PAW OEDEMA IN RATS

Experimental design

Sprague Dawley rats weighed around 120-200g were used for the study. Rats were divided into five groups of 6 animals each.

Table: 2 Experimental design for carrageenan induced paw oedema

GROUPS	TREATMENT
Group I	Carrageenan 1%w/v (0.1 ml, sub plantar)
Group II	Diclofenac(5 mg/kg) i.p
Group III	GNP (30 mg/kg)
Group IV	GNP (60 mg/kg)
Group V	GCS(60 µg/100 g)

Procedure [73-76]

The rats were divided into five groups of six animals each. The group I was treated with freshly prepared 0.1 ml carrageenan, group II was treated with Diclofenac (5 mg/kg) i.p, group III and IV were treated with GNP 30 mg/kg and 60 mg/kg respectively. The group V treated with GCS (60 µg/100 gm). Treatments were given 30 min before the administration of carrageenan. The rats were then challenged with injection of 0.1 ml of 1%w/v solution of Carrageenan into the sub plantar region of left paw. The paw was marked with ink at the level of lateral malleolus. The paw volume was measured before (0 h) and after carrageenan injection at 1, 2, 3, 4, 5 and 6 h by volume displacement method using Plethysmometer. The difference of average values between treated animals and control group is calculated for each time interval and evaluated statistically. The percentage inhibition (PI) of paw edema was calculated by using the following formula;

Percentage of edema inhibition = {(Vc –Vt)/Vc} × 100

Vc- Volume of paw oedema in control group.

Vt- Volume of paw oedema in treated groups.

5.4.7.2. HISTAMINE INDUCED RAT PAW OEDEMA

Experimental design

Sprague Dawley rats were divided into five groups of 6 animals each.

Table: 3 Experimental design for histamine induced paw oedema

GROUPS	TREATMENT
Group I	0.1 ml of freshly prepared histamine (1%)
Group II	Indomethacin (10 mg/kg) i.p
Group III	GNP (30 mg/kg)
Group IV	GNP (60 mg/kg)
Group V	GCS (60 µg/100 g)

Procedure [77]

30 min after the drug treatment, inflammation was induced by injection of freshly prepared histamine (0.1 ml) in normal saline underneath the plantar tissue of the right hind paw of rats. Paw volume was measured using a plethysmometer before histamine administration and at 1, 2, and 3 h after histamine injection. The percentage inhibition (PI) of paw oedema was calculated by using the following formula;

Percentage of edema inhibition = {(Vc –Vt)/Vc} × 100

Vc- Volume of paw edema in control group.

Vt- Volume of paw edema in treated groups.

5.4.7.3. SEROTONIN INDUCED RAT PAW OEDEMA

Experimental design

Sprague Dawley rats were divided into five groups of 6 animals each.

Table: 4 Experimental design for serotonin induced paw oedema

GROUPS	TREATMENT
Group I	0.1 ml of freshly prepared Serotonin (1%)
Group II	Indomethacin (10 mg/kg) i.p
Group III	GNP (30 mg/kg)
Group IV	GNP (60 mg/kg)
Group V	GCS (60 µg/100 g)

Procedure [78]

30 min after the drug treatment, inflammation was induced by injection of 0.1 ml of freshly prepared serotonin (1%) in normal saline underneath the plantar tissue of the right hind paw of rats. Paw volume, measured using a plethysmometer before serotonin administration and at 1, 2, and 3 h after histamine injection. The percentage inhibition (PI) of paw edema was calculated by using the following formula;

Percentage of edema inhibition = {(Vc –Vt)/Vc} × 100

Vc- Volume of paw edema in control group.

Vt- Volume of paw edema in treated groups.

5.5. STATISTICAL ANALYSIS

Data were analyzed by one way ANOVA followed by Dunnetts's/Tukey's multiple comparison tests using Graphpad 5.0 software. The values were expressed as Mean±SEM.

6. RESULTS

6.1. Qualitative analysis of GNP and GOLD Colloid solution

6.1.1. SEM image and XRD value of GOLD NANOPARTICLES

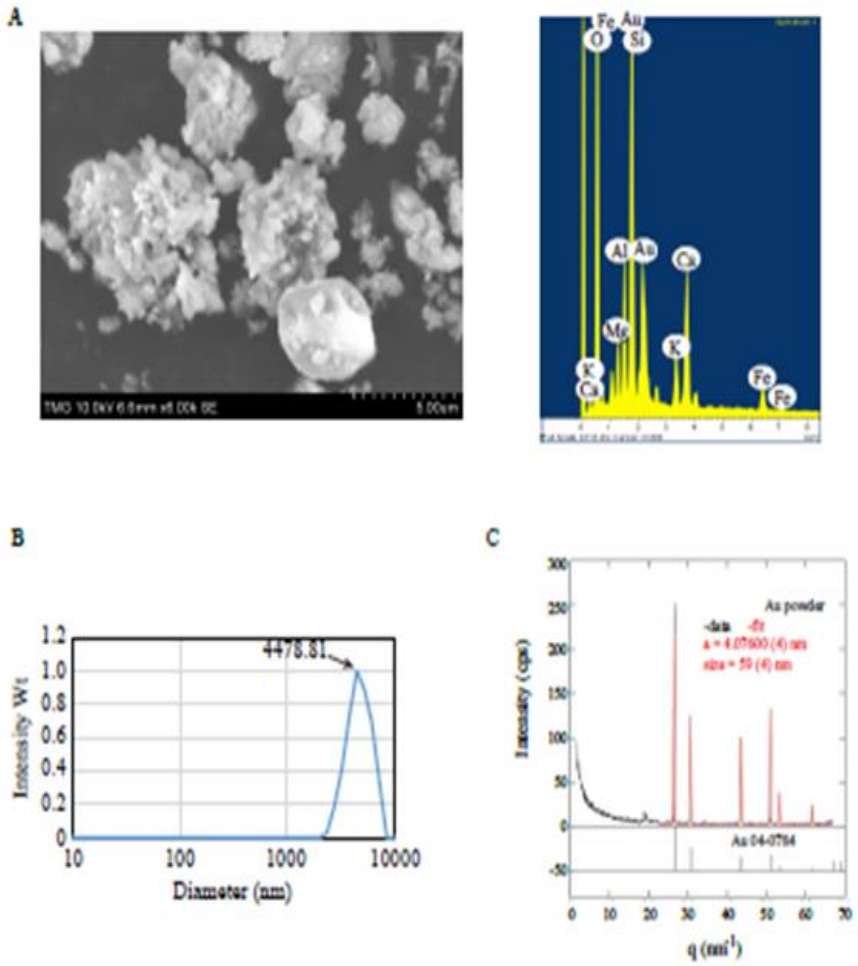


Figure. 3 A) GNP were imaged by SEM(left) and by EDS –SEM(right), which shows the shape and size of the particles, and the elemental composition, respectively.

B) A graph shows the mean size of the GNP by DLS.

C) A graph shows the XRD pattern of GNP in comparison to gold and the size of individual particles is indicated.

6.1.2. SEM images and XRD values of GOLD COLLOID SOLUTION

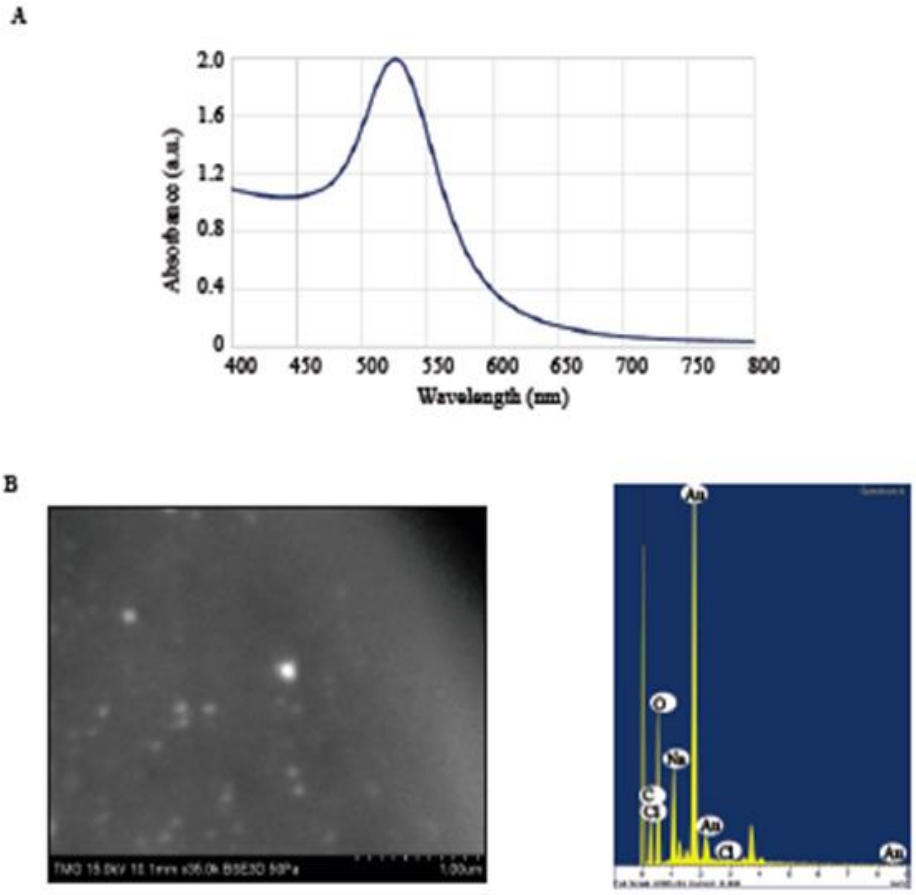


Figure. 4 A) The graph shows the LSPR band corresponding to gold colloid solution.

B) Gold colloid solution were imaged by SEM (left) and by EDS–SEM (right), which shows the shape and size of the particles and the elemental composition respectively.

Table: 5 ELEMENTAL COMPOSITIONS OF GOLD NANOPARTICLES

Elements	Concentration %
Gold (Au)	56.88
Mg	1.8
Ca	1.4
Fe	0.29
Si	0.29
Trace Elements	
Mn	0.037
Ni	0.02
As	0.15

Table: 6 ELEMENTAL COMPOSITION OF COLLOIDAL GOLD

Elements	Concentration (ppm)
Gold (Au)	89.6
Mg	0.273
Ca	1.16
Na	20.9
Si	2.69

6.1.3. DLS (Dynamic light scattering)

- Average size of Gold nanoparticles- 4.4 nm
- Average size of Colloidal gold- 31 nm

6.2. MTT ASSAY

Table: 7 Cytotoxic activity of GNP and GCS in HeLa cell line

Compound	Cell line	Concentration (µL)	% Cell Growth
GNP	HeLa	12.5	100.8065
		25	100.1241
		50	99.6898
		100	94.4789
		200	83.4987
GCS	HeLa	12.5	101.1166
		25	99.9379
		50	99.5037
		100	94.6029
		200	89.2059

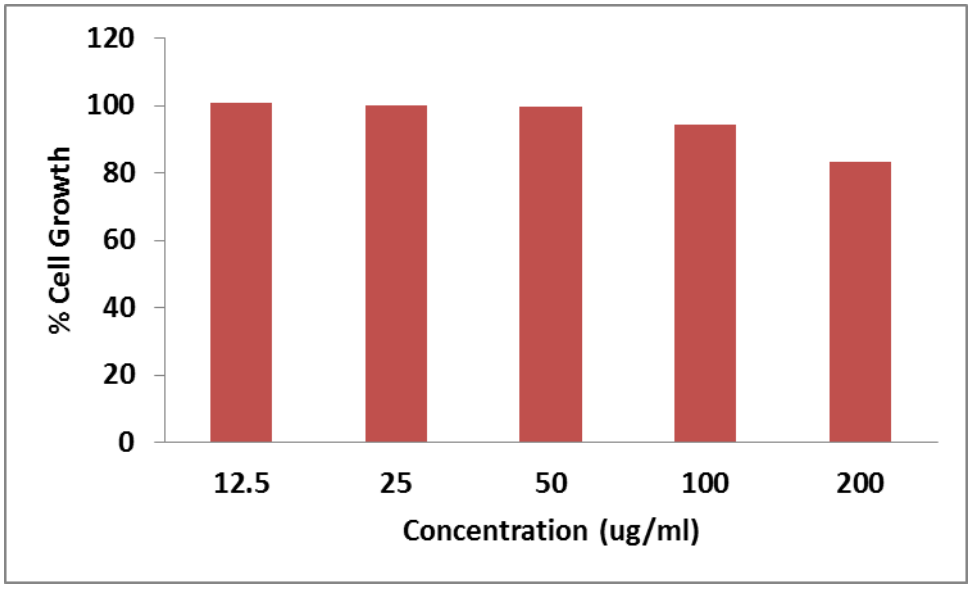


Figure: 5 Graphical representation of Cytotoxic activity of GNP in HeLa Cell line

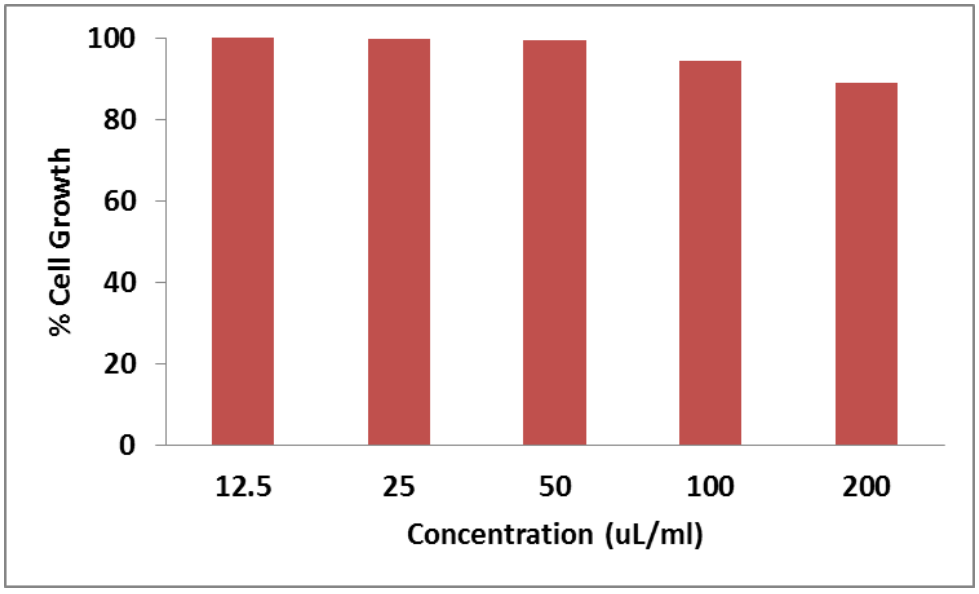
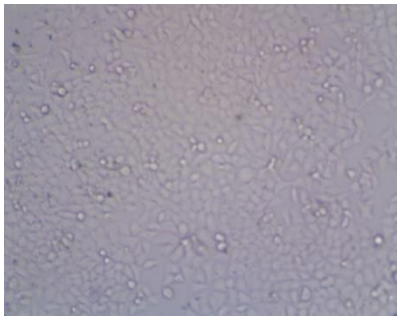
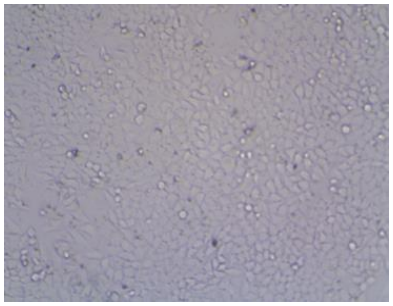


Figure: 6 Graphical representation of Cytotoxic activity of GCS in HeLa cell line

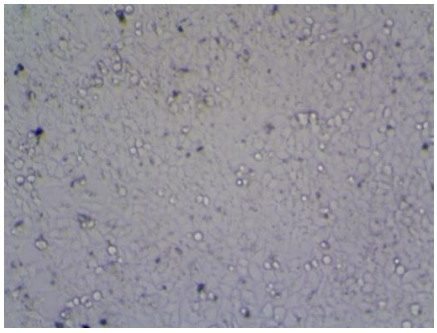
Figure: 7 Pictoral view of Cytotoxic activity of GNP in HeLa Cell line



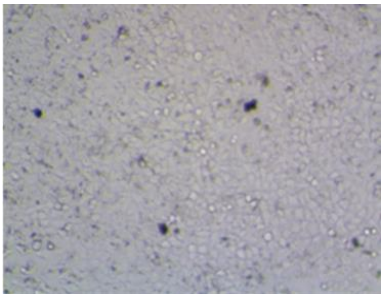
12.5 µL



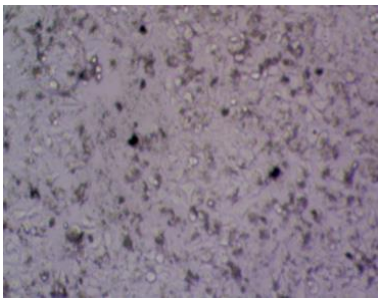
25 µL



50 µL

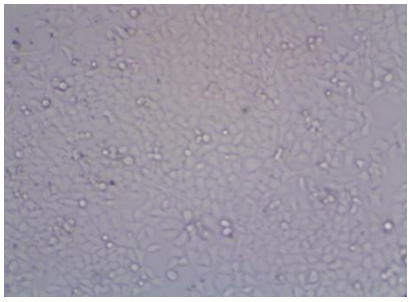


100 µL

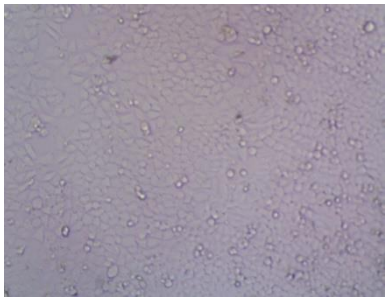


200 µL

Figure: 8 Pictoral view of Cytotoxic activity of GCS in HeLa Cell line

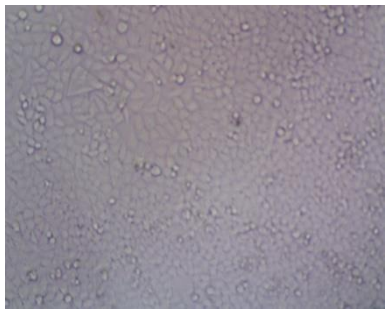


12.5 µL

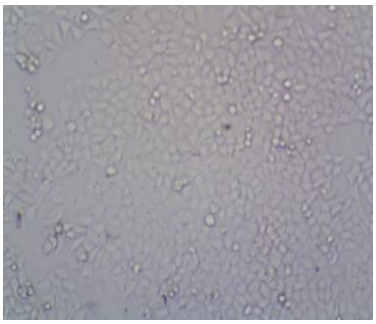


25 µL

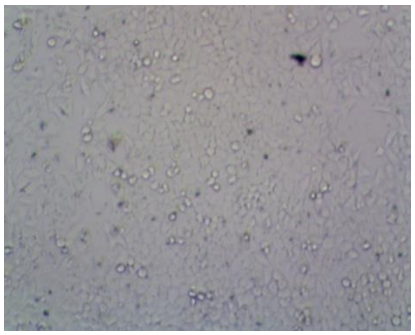
25



50 µL



100 µL

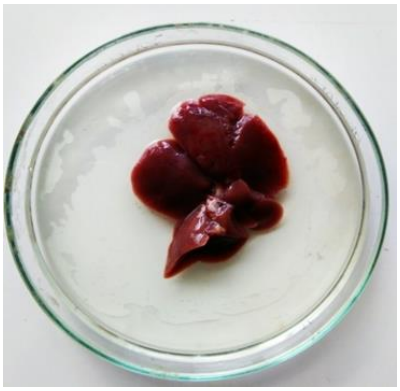


200 µL

6.3. HCC DEVELOPMENT

Normal

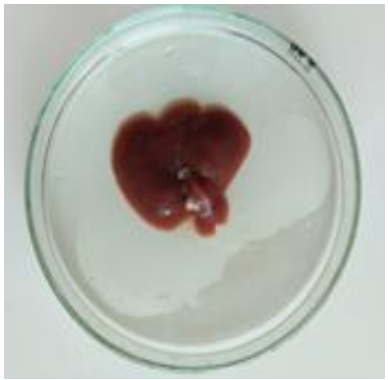
Control



Standard

GNP Low dose

GNP High dose



Gold Colloid Solution

Figure: 9 Development of Tumor in experimental rats

6.4. PHARMACOLOGICAL EVALUATION

6.4.1. ESTIMATION OF HEMATOLOGICAL PARAMETERS

Table: 8 Effect of GNP& GCS on the RBC& WBC counts

Groups	Normal	Control	Standard	GNP Low dose	GNP High dose	GCS
RBC (1 x 10^6cells/mm^3)	9.97±0.13	5.29±0.08***	6.82±0.06***	8.65±0.13***	7.83±0.25***	8.40±0.29***
WBC (1 x 10^3cells/mm^3)	5.06±0.12	5.86±0.16***	6.46±0.14*	3.01±0.09***	5.36±0.12ns	4.94±0.03***

***- P<0.001, **- P<0.01, *- P<0.05, ns – Non Significant

Data is expressed as Mean ± SEM (n = 6 animals in each group)

Analysis of Variance (ANOVA); one way ANOVA followed by Tukey:Compare Test was performed. The hepatocellular control group was compared with normal and all other groups were compared with hepatocellular control group. The value shows significant at P<0.001.

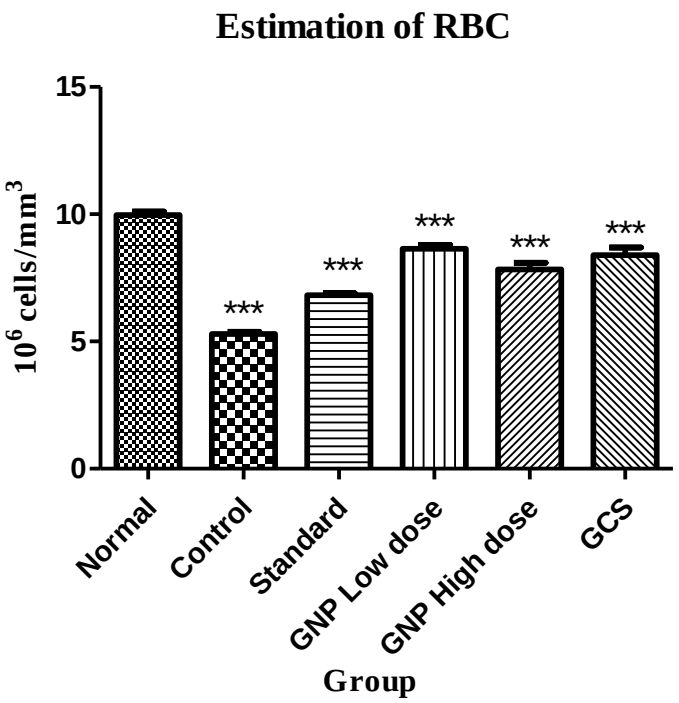


Figure: 10 Estimation of RBC

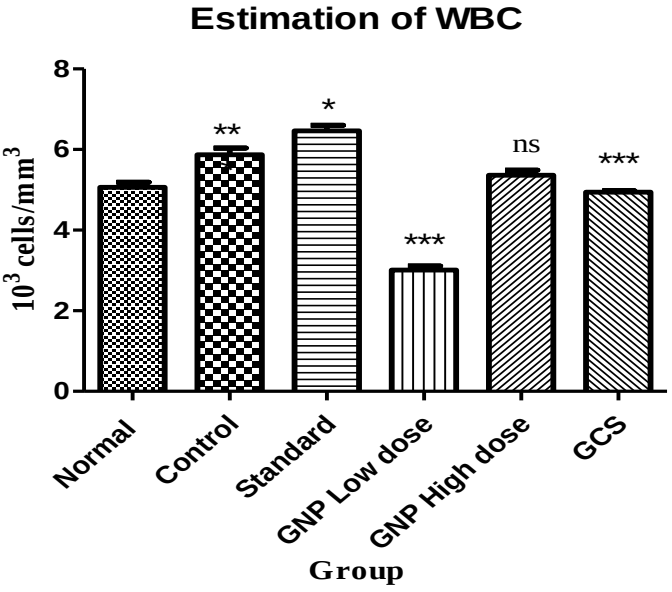


Figure: 11 Estimation of WBC

6.4.2. BODY WEIGHT ANALYSIS

Table: 9 Effect of the GNP& GCS on the Body weight of the Experimental rats

Weight in gram	Normal	Control	Standard	GNP Low dose	GNP High dose	GCS
Initial body weight	209.5±2.2	151.5±2.2***	143.5±2.2***	119.7±1.2***	108.2±1.4***	125.8±1.7***
Final body weight	337.5±2.2	287.0±1.5***	258.0±1.5***	225.2±1.7***	200.5±2.3***	213.5±0.4***

***- P<0.001, **- P<0.01, *- P<0.05, ns – Non Significant

Data is expressed as Mean ± SEM (n = 6 animals in each group)

Analysis of Variance (ANOVA); one way ANOVA followed by Tukey:Compare Test was performed. The hepatocellular control group was compared with normal and all other groups were compared with hepatocellular control group. The value shows significant at P< 0.001.

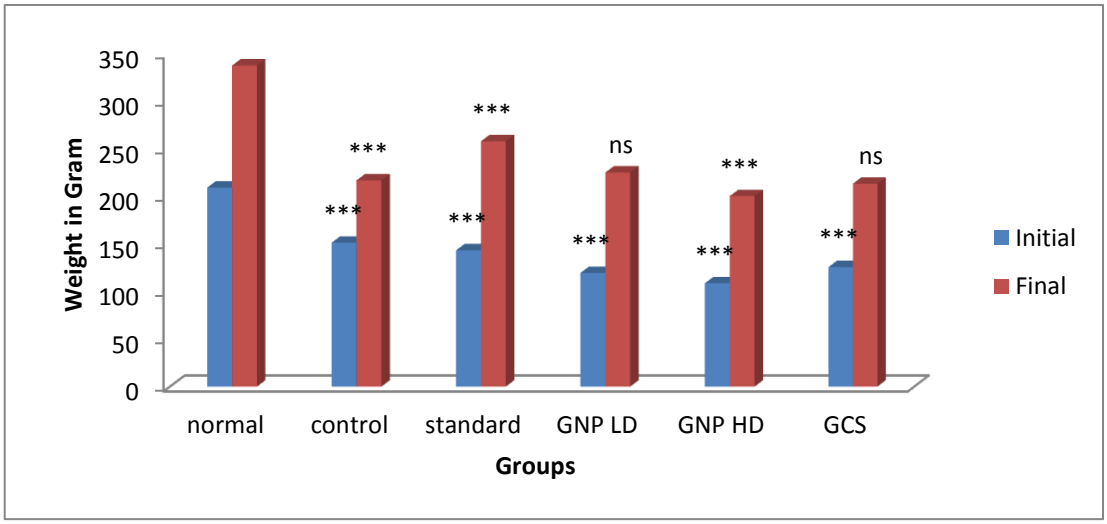


Figure: 12 Estimation of Body weight analysis

6.4.3. LIVER WEIGHT ANALYSIS

Table: 10 Effect of the GNP& GCS on the Liver weight of the Experimental rats

Groups	Normal	Control	Standard	GNP Low dose	GNP High dose	GCS
Liver Weight (in gm)	8.7±0.13	10.7±0.15**	9.4±0.24***	9.5±0.14**	9.8±0.23*	9.3±0.17***

***- P<0.001, **- P<0.01, *- P<0.05, ns – Non Significant

Data is expressed as Mean ± SEM (n = 6 animals in each group)

Analysis of Variance (ANOVA); one way ANOVA followed by Tukey:Compare Test was performed. The hepatocellular control group was compared with normal and all other groups were compared with hepatocellular control group. The value shows significant at P< 0.001.

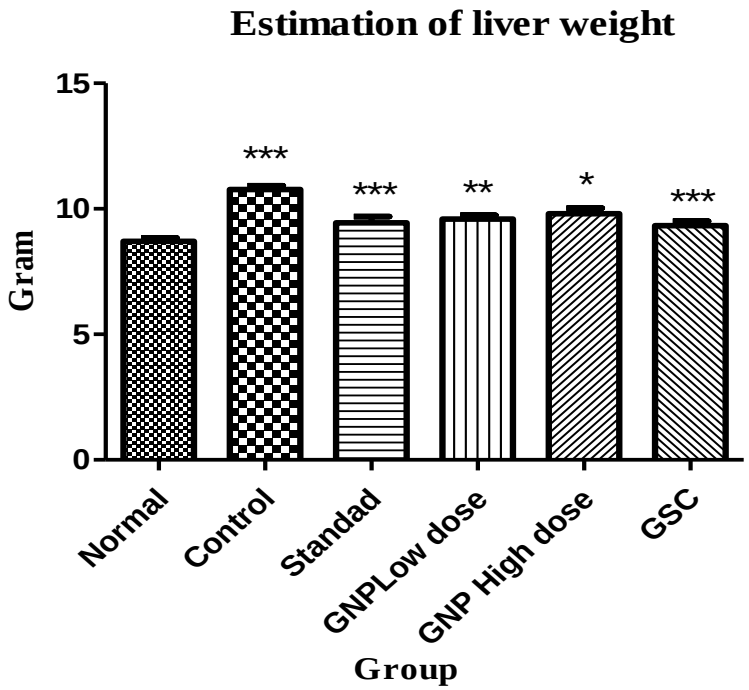


Figure: 13 Liver weight analysis

6.4.4. SERUM BIOCHEMICAL PARAMETERS

Table: 11 Effect of the GNP& GCS on the Serum biochemical parameter

Groups	SGOT(U/L)	SGPT(U/L)	ALP(U/L)	Total protein(g/dl)
Normal	35.18±0.625	39.91±1.151	122.1±0.832	4.058±0.112
Control	66.67±0.763***	74.66±2.753***	143.1±3.537***	6.33±0.118***
Standard	59.33±0.844***	102.8±1.563**	179.5±3.217***	5.068±0.118***
GNP low dose	63.39±0.420*	125.9±2.957**	165.2±5.048***	6.964±0.145*
GNP high dose	61.84±0.649***	118.0±7.593***	150.2±3.225ns	7.080±0.120**
GCS	62.04±0.319***	121.4±5.318***	153.9±0.416ns	6.898±0.120***

***- P<0.001, **- P<0.01, *- P<0.05, ns – Non Significant

Data is expressed as Mean ± SEM (n = 6 animals in each group)

Analysis of Variance (ANOVA); one way ANOVA followed by Tukey:Compare Test was performed. The hepatocellular control group was compared with normal and all other groups were compared with hepatocellular control group. The value shows significant at P< 0.001.

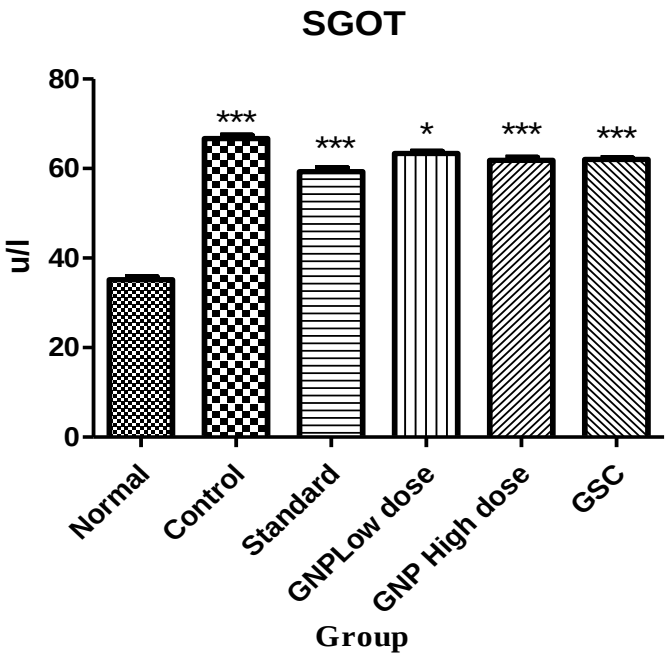


Figure: 14 Effect of GNP and GCS in serum SGOT level

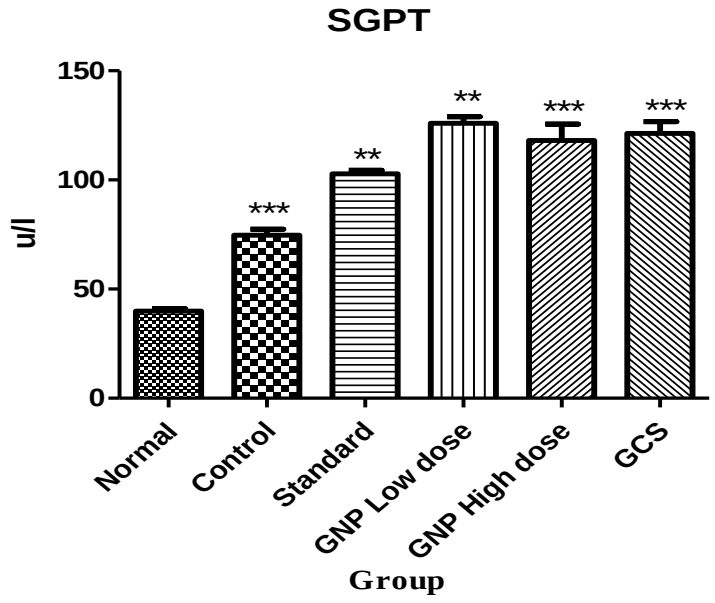


Figure: 15 Effect of GNP and GCS in serum SGPT level

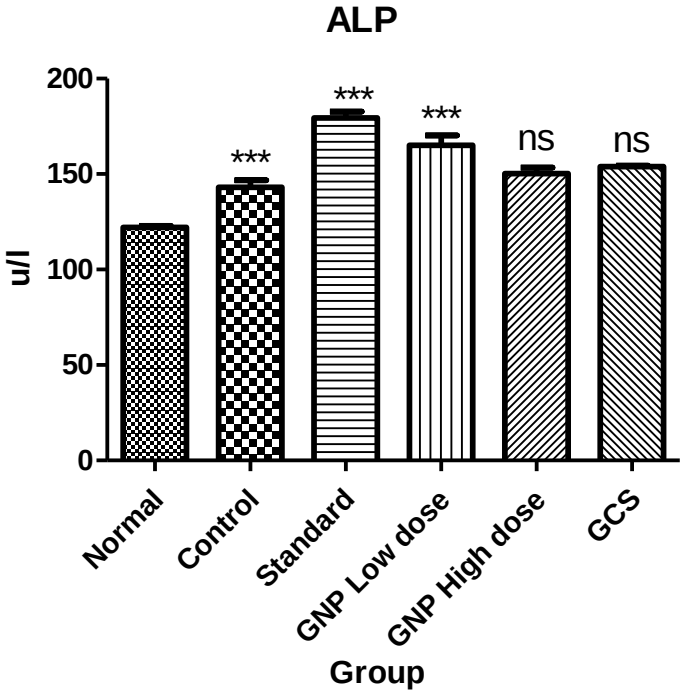


Figure: 16 Estimation of GNP and GCS in Serum ALP level

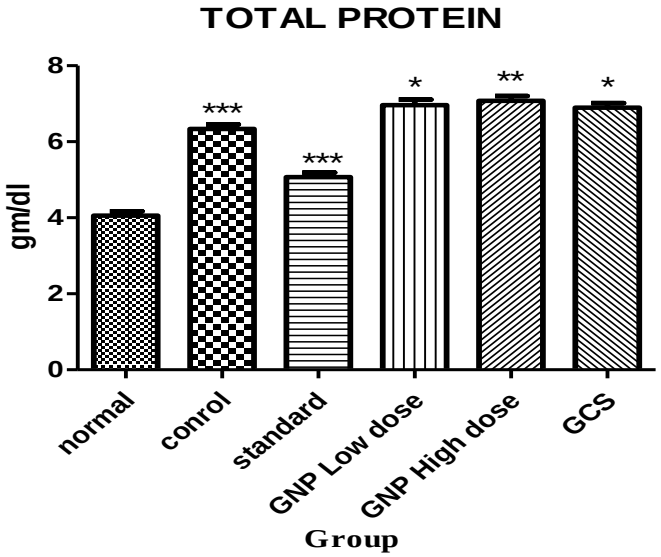
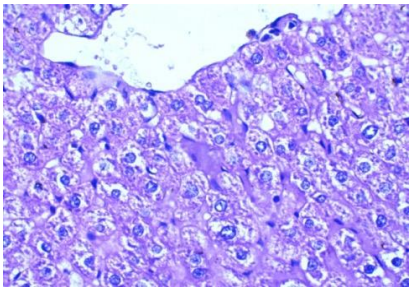


Figure: 17 Estimation of GNP and GCS on Serum TOTAL PROTEIN level

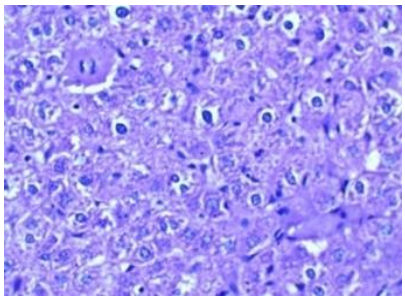
6.5. ESTIMATION OF HISTOPATHOLOGY

Normal



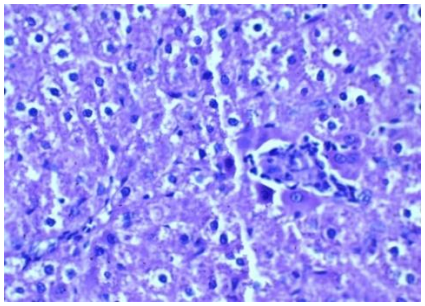
Architecture effaced
Cells-Enlarged, Granularoty-Present
Cellularity atypia and Mitotic activity-present

Control



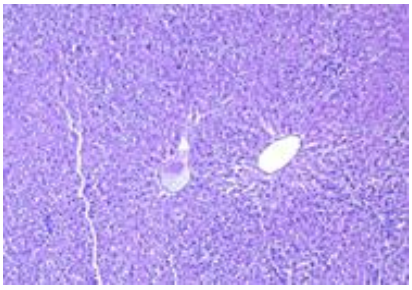
Architecture-Normal
Cells- Unremarkable

GNP Low dose



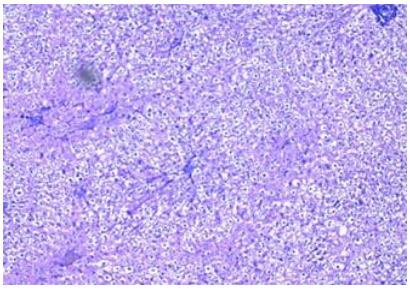
Liver architecture midly effaced
Cytoplasmic granularity-Present, Nuclei-Enlarged
Few apoptotic bodies -few

GNP High dose



Architecture- preserved
Cytoplasm- Granularity moderately reduced
Nuclei- Normal

Gold Colloid Solution



Architecture- Preserved
Cytoplasmic granularity-Markedlyreduced
Nuclei-Normal

Figure: 18 Estimation of histopathology

6.6. SCREENING OF ANTI-INFLAMMATORY ACTIVITY

6.6.1. CARRAGEENAN INDUCED PAW OEDEMA IN RATS

Table: 12 Effect of EECT on carrageenan induced paw oedema in rats

GROUP	TREATMENT	Mean oedema volume (ml) and % inhibition					
		1 h	2 h	3 h	4 h	5 h	6 h
1	Carrageenan 1%w/v (0.1ml)	0.0475±0.004	0.0800±0.004	0.1275±0.010	0.1650±0.013	0.1700±0.012	0.177±0.014
II	Diclofenac (5mg/kg)	0.032±0.06 ns 31.57%	0.051±0.006 * 35.75%	0.077±0.010 * 39.21%	0.085±0.010 ** 48.48%	0.082±0.013 ** 51.45%	0.085±0.015** 52.11%
III	GNP (30mg/kg)	0.042±0.001 ns 11.57%	0.059±0.008 ** 25.62%	0.089±0.008 * 30.03%	0.110±0.007 * 33.33%	0.115±0.006 ** 32.41%	0.125±0.012 ** 30.70%
I V	GNP (60 mg/kg)	0.035±0.002 * 23.15%	0.053±0.002 ** 33.12%	0.079±0.002 ** 37.88%	0.089±0.002 ** 45.51%	0.094±0.005 ** 44.29%	0.107±0.004 ** 43.66%
V	GCS (60µg/100g)	0.038±0.00 ns 18.52%	0.057±0.001 * 28.72%	0.082±0.001 ** 35.68%	0.099±0.004 ** 39.87%	0.107±0.004 ** 38.23%	0.110±0.007 ** 38.02%

Statistical comparison: Values represent mean ± SEM, n=6, statistical analysis was done by one way analysis of variation (ANOVA) followed by Tukey:compare test.

***P<0.001, **P<0.01, *P<0.05 and ns- non significant denote the comparison of treated groups with control

6.6.2. HISTAMINE INDUCED PAW OEDEMA IN RATS

Table: 13 Effect of GNP and GCS on Histamine induced paw oedema in rats

Group	TREATMENT	Mean oedema volume (ml) and % inhibition		
		1 hr	2hr	3hr
I	Histamine 1%w/v (0.1ml)	0.0700±0.007	0.1350±0.0155	0.1600±0.016
II	Indomethacin(25mg/kg)	0.0375±0.006* 46.42%	0.0550±0.008** 59.25%	0.0550±0.005*** 65.62%
III	GNP(30mg/kg)	0.0450±0.006* 35.71%	0.675±0.006** 50%	0.0600±0.007** 62.5%
I	GNP(60 mg/kg)	0.0550±0.006ns 21.42%	0.0900±0.007* 33.33%	0.0850±0.005*** 46.87%
V	GCS(60µg/100g))	0.0450±0.005* 35.71%	0.0650±0.008** 51.85%	0.0575±0.004** 64.06%

Statistical comparison: Values represent mean ± SEM, n=6, statistical analysis was done by one way analysis of variation (ANOVA) followed by Tukey:compare test.

***P<0.001, **P<0.01, *P<0.05 and ns- non significant denote the comparison of treated groups with control.

6.6.3. SEROTONIN INDUCED PAW OEDEMA

Table: 14 Effect of GNP and GCS on serotonin induced paw oedema in rats

Group	TREATMENT	Mean oedema volume (ml) and % inhibition		
		1 hr	2hr	3hr
I	serotonin 1%w/v (0.1ml)	0.0775±0.02	0.1100±0.002	0.1375±0.001
II	Indomethacin(25mg/kg)	0.0605±0.002*** 21.93%	0.0700±0.0008*** 36.36%	0.0752±0.001*** 45.30%
III	GNP(30mg/kg)	0.0615±0.0006*** 20.64%	0.0750±0.009ns 31.81%	0.0820±0.0008*** 40.36%
I V	GNP(60 mg/kg)	0.0620±0.001** 20%	0.0775±0.006** 29.54%	0.0950±0.001** 30.90%
V	GCS(60µg/100g))	0.0650±0.0008** 16.12%	0.0850±0.0008* 22.72%	0.1±0.003** 27.27%

Statistical comparison: Values represent mean ± SEM, n=6, statistical analysis was done by one way analysis of variation (ANOVA) followed by Tukey:compare test.

***P<0.001, **P<0.01, *P<0.05 and ns- non significant denote the comparison of treated groups with control.

7. DISCUSSION

FESEM revealed the structure of the Gold nanoparticles and Gold colloid solution. It showed nano-scale structure of gold nano particles. When reduced to this size any medicinal compound gains additional properties such as better bioavailability, lower dose requirement, target specificity etc.

Dynamic light scattering (DLS) measures the particle size of Gold nanoparticles and Gold colloid solution. The particle size of Gold nanoparticles and Gold colloid solution shows 4.4 nm and 31 nm respectively.

The XRD pattern of Gold nanoparticles and Gold colloid solution reflects gold metal as the major phase along with certain impurities. The EDS reports of Gold nanoparticles and Gold colloid solution show peaks for Mg, Ca, and Si these elements are common to found in both preparations.

The *in vitro* anti-cancer activities of GNP and GCS in MTT assay were determined by using HeLa cell lines. The MTT assay is based on metabolic reduction of dye MTT to water soluble blue formazan complex is directly proportional to number of visible cells Nano particles did not be show any toxic effect in isolated cells. As per reported by many other studies, [79] gold nanoparticles enters the cell by (presumably) endocytosis, did not induce any toxicity, and reduce the level of reactive oxygen species. Toxicity of gold nanoparticles (spheres, 10 nm in diameter) when studied on dendritic cells (part of the human immune system, which process and present antigens on their surfaces for other cells) nanoparticles were found to be non-cytotoxic, and it did not induce activation or didn't change phenotype of the cells. [80] . In the present study ,in *in vitro* condition GNP and GCS do not produce any cytotoxic effect.

The formation of reactive oxygen species (ROS) is deceptive during the metabolic biotransformation of DEN resulting in oxidative stress. Oxidative stress leads to carcinogenesis by several mechanisms including the damage of integrity of various biomolecules including DNA, lipid and protein, change in intracellular signaling pathways and even changes in gene expression. Lipid peroxidation (LPO)

may possibly also result in several changes, including structural and functional membrane modifications, protein oxidation and generation of oxidation products such as acrolein, crotonaldehyde, malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), which are considered strong carcinogens. [81]

In the current study, a significant reduction in the body weight of the diseased animals was observed when compared with the normal control. The GNP and GCS treated group has showed an average increased body weight that was comparable with the standard group as well as the normal control group. The GNP and GCS group has shown increased body weight than the diseased control group.

Increase in liver weight was observed for the disease control group and the marked reduction of weight was observable in both GNP and GCS treated groups.

Alterations were seen in the haematological parameters in HCC. The disease control group has shown a significant reduction in RBC and elevation of WBC. The GNP and GCS group has shown a significant increase of RBC. The control group showed significant increase in WBC count while the GNP and GCS treated group showed moderate reduction in WBC.

Serum biochemical parameters show an altered pattern in the cancerous condition. The escalation in the activities of plasma ALP, SGOT, SGPT indicated that DEN may prompt hepatic dysfunction. The enzymes directly associated with the conversion of amino acids to keto acids are AST and ALT, and are proved to be increased in the HCC condition. [82] All the biochemical parameters were significantly elevated in the disease control group. GNP and GCS treatment group was also very effective in normalizing the SGPT, SGOT level, but only a moderately significant reduction was shown for the other parameters.

Total protein present in the liver tissue of disease control group was reduced due to the cancerous condition. But in the GNP and GCS treated group there was a significant increase in the protein levels compared to disease control and no noticeable difference when compared to the normal control.

The results obtained from the current study demonstrate that the GNP and GCS groups showed higher shielding in DEN induced HCC rat model at 30mg/kg.

The anti-inflammatory activity of the GNP and GCS was evaluated by three experimental models, i.e., carrageenan-induced paw oedema, Histamine induced paw oedema and serotonin induced paw oedema. Carrageenan induced paw oedema model is used to screen the anti-inflammatory activity of a drug in the acute phase of inflammation. Sub plantar injection of carrageenan into the rat paw produces plasma extravasation and the inflammation characterized by increased tissue water and plasma protein exudation with neutrophil extravasation and metabolism of arachidonic acid by both cyclooxygenase and lipo oxygenase enzyme pathways. Oedema induced by carrageenan is a biphasic event. The first phase (1 hour) involves the release of serotonin and histamine and the second phase (> 1 hour) is mediated by prostaglandins. The mean oedema volume and percentage inhibition was calculated for 6 hours.

In carrageenan induced paw oedema, the percentage inhibition of GNP (30mg/kg) was found to be 11.57%, 25.62%, 30.03%, 33.33%, 32.41% and 30.70%. at 1 h to 6 h respectively, GNP (60 mg/kg) was found to be 23.15%, 33.12%, 37.88%, 45.51%, 44.29% and 43.66% at 1 h to 6 h respectively. GCS (60 µg/100 g) was found to be 18.52%, 28.72%, 35.68%, 39.87%, 38.23% and 38.02% at 1 h to 6 h respectively.

The highest percentage inhibition for GNP (30 mg/kg) was found to be at 4^{th} h (33.33%), GNP (60 mg/kg) at 4^{th} h (45.51%) and GCS (60 µg/100 g) at 4^{th} h (39.87%).

A significant anti oedematous activity of both doses of GNP (30 mg/kg and 60 mg/kg) and GCS (60 µg/100 g) was observed during the second phase of inflammation, indicating the inhibition of prostaglandin release. The maximal percentage inhibition was observed at 3^{rd} and 4^{th} hours.

It may be concluded that GNP (60mg/kg) significantly suppressed 45.51 % paw oedema from 4h shows higher activity. So it might be blocking prostaglandin and /or bradykinin release rather than histamine and/or serotonin. Diclofenac also has shown similar effect only at second phase.

The two doses of formulation GNP (30 mg/kg),GNP(60 mg/kg) and GCS (60µg/100 g) exerted a significant inhibition of 35.71%, 21.42% and 35.71% at 1 h

and 50%, 33.33% and 51.85% at 2 h and 62.5%, 46.87% and 64.06% at 3 h respectively in the histamine induced rat paw oedema model. It was observed that the GNP and GCS was capable of inhibiting oedema induced by histamine and the effectiveness for suppression of oedema might be due to the ability of GNP and GCS to inhibit the synthesis, release or action of histamine involved in the inflammation. GCS (60 µg/100 g) significantly suppressed 64.06% paw oedema at 3 h shows higher activity.

The two doses of formulation GNP (30 mg/kg and 60 mg/kg) and GCS (60µg/100 g) exerted a significant inhibition of 20.64%, 20% and 16.12% at 1 h and 31.81%, 29.54% and 22.72% at 2 h and 40.36%, 30.90% and 27.27% at 3 h respectively in the serotonin induced rat paw oedema model . It was observed that the GNP and GCS were capable of inhibiting oedema induced by serotonin. GNP (30mg/kg) significantly suppressed 40.36% at 3 h shows higher activity.

Molecular and cellular pathways shows link with inflammation and cancer. Schematically, there are two pathways: intrinsic and extrinsic. In the extrinsic pathway, inflammatory conditions promote cancer development. Inflammatory mediators destabilize the cancer cell genome which accelerates the somatic evolution of cancer.

COX-2, a key isoenzyme in conversion of arachidonic acid to prostaglandins is inducible by various agents such as growth factors and tumor promoters and frequently over expressed in various tumors. The potency of COX-2 inhibitors in vivo can be attributed to the inhibition of the enzyme in the tumour as well as in stromal cells, resulting in anti-proliferative and pro-apoptopic actions within the tumour, and anti-angiogenic and pro-immune surveillance activities in endothelial and myeloid cells. The combination of COX-2 inhibitor with standard cancer chemotherapeutic and/or radiation may provide additional therapeutic paradigms in the treatment of various human cancers. Hence, the cyclooxygenase-2 (COX-2) has a role in hepatocarcinogenesis, as selective COX-2 inhibitors (COXIBs) show antiproliferative and pro-apoptotic effects in human HCC cell lines. [83]

Besides this transcription factor, essential in regulating inflammation and cancer development, an inflammatory microenvironment inhabiting various inflammatory cells and a network of signaling molecules are indispensable for the

malignant progression of transformed cells, which is attributed to the mutagenic predisposition of persistent infection fighting agents at sites of chronic inflammation. [81 The contribution of COX-2 is being related to its abilities to increase the production of prostaglandins, convert procarcinogens in to carcinogen, inhibit apoptosis promote angiogenesis modulate inflammation and immune function and increase tumor cell invasiveness. COX-2 inhibitors show marked inhibition of tumor growth and metastasis. [84]

The potential of the compound to inhibit cyclooxygenase enzyme drive us to the assumption that the anticancer activity of GNP and GCS may be associated with the inhibition of COX-2 enzyme. Further studies are required to confirm this.

8. CONCLUSION

In the present study GNP (Thangaparpam) and GCS (Gold colloid solution) were evaluated for its anti-inflammatory and anti cancer activities. Qualitative analysis of GNP and GCS performed by various methods like, FESEM, DLS, XRD and EDS showed differences in particle size, surface property, and elemental composition. GNP and GCS showed significant anti-inflammatory activity in carrageenan, histamine and serotonin induced paw edema methods compared to the respective standard drugs. On the other hand, both the compounds showed remarkable anti cancer activity in the *in vitro* and *in vivo* models (ie,MTT assay and DEN induced HCC model respectively)

Persistent inflammation is known to promote and exacerbate malignancy. The inflammatory mediators, some of that are direct mutagens, also directly or indirectly down regulate DNA repair pathways and cell cycle checkpoints, thus destabilizing cancer cell genome and contributing to the accumulation of random genetic alterations which in turn accelerate the somatic evolution of cancer. Hepatocellular carcinoma (HCC), is a clear example of inflammation-related cancer as more than 90 % of HCCs arise in the context of hepatic injury and inflammation. This is suggestive for the mechanism behind remarkable anticancer activity obtained for GNP and GCS in the present study. Already well known for its anti inflammatory activity, these formulations can further evolve as an potent anticancer agent or can be used in combination with other anticancer drugs with the goal of improving the efficiency of the anti-cancer protocol. Further studies are required to validate the anticancer activity against HCC and to evaluate its effectiveness in other types of cancer.

ABSTRACT

Aim: This study aims to elucidate the *in vivo*, *in vitro* anti-cancer and anti-inflammatory effects of GNP and GCS.

Methodology: The qualitative analysis of GNP and GCS were performed by different methods, i. e. FESEM, DLS, XRD, EDS.

In vitro anticancer study performed by MTT assay. The cytotoxic activity of GNP and GCS at different concentrations (12.5, 25, 50, 100 and 200 µL) were determined using HeLa cell line.

In *in vivo* anticancer study, initiation of HCC was done by single i.p injection of DEN at a dose of 200mg/kg. The GNP (30 mg/kg, 60 mg/kg) and GCS (60 µg/100 kg) received treatment for 90 days after 7 days of development of HCC and continued for entire study period, whereas the other two groups given normal saline and 5-flurouracil (20mg/kg) i.p.

The anti-inflammatory activity of the GNP (30 mg/kg, 60 mg/kg) and GCS (60 µg/100kg) were evaluated by three experimental models, i.e. Carrageenan-induced paw edema model, in which diclofenac (5mg/kg) was used as standard. Whereas in histamine and serotonin induced paw edema models indomethacin (10mg/kg) was the standard.

Results: The results showed that the injection DEN lead to the development of liver tumors in rats. Significant elevation of the serum biochemical parameters like ALP, SGOT, SGPT and Total protein. The results exhibited that GNP and GCS treatment group have excellent shielding against HCC and displayed all the parameters in near normal range. GNP and GCS also exhibited significant anti-inflammatory activity which was evident with reduction in mean paw oedema volume in carrageenan, histamine and serotonin induced inflammatory models.

Conclusion: The results revealed that the GNP and GCS possess significant inhibition of paw oedema in all the three inflammatory models.These formulations can further evolve as an potent anticancer agent or can be used in combination with other anticancer drugs with the goal of improving the efficiency of the anti-cancer protocol. Further studies are required to validate the anticancer activity against HCC and to evaluate its effectiveness in other types of cancer.

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